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Bacteriophage efficacy in controlling bacterial leaf spot disease on water spinach (*Ipomoea aquatica)*

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ABSTRACT

Bacterial leaf spot disease on water spinach (Ipomoea aquatica) is common and causes severe defoliation. The bacterial pathogen responsible for bacterial leaf spot disease in water spinach was identified as Xanthomonas sp. using biochemical methods and the 16S rRNA-DNA region. In addition, this study further evaluates bacteriophages as a novel biological control approach. From twelve isolated bacteriophages, two with the largest plaque diameters, Φ8b and Φ9, were selected for evaluation of their efficacy in disease control. Results from both net house and field trials showed that these two bacteriophages, when used individually, significantly reduced disease incidence and percentage of infected leaf area compared to the control. However, the mixture of bacteriophages did not present a clear disease control effect. This study demonstrates the potential of bacteriophages for managing bacterial leaf spot disease in water spinach.

1. INTRODUCTION

Water spinach (*Ipomoea aquatica*) constitutes a common component of the Vietnamese diet (Duc et al., 1999; Marcussen et al., 2008). Within the Mekong Delta region, water spinach is extensively cultivated across provinces. Despite its widespread cultivation, no advanced agricultural techniques are implemented, resulting in low-level farming practices and significant levels of damage by pests and diseases. Among these, bacterial leaf spots have emerged as a significant threat to water spinach plants. Bacterial leaf spot on water spinach has been reported to be caused by *Xanthomonas* sp., identified by its yellow pigmentation and xanthomonad in production. The disease was first observed in 1990 in Bang Pai, Bangkok, Thailand, where it caused significant yield loss (Leksomboon & Thaveechai, 1991).

Preliminary in-depth interviews conducted among five farmers in Thoi An ward, O Mon district, Can Tho city, revealed that the disease is prevalent during the rainy season and poses challenges in prevention and control. Farmers often resort to chemical treatments as the primary control measure when the disease manifests. However, the indiscriminate use of chemicals can potentially pose significant risks to both human health and the environment (Dasgupta et al., 2007; Ansari et al., 2014). To mitigate these risks and promote sustainable agriculture, there is a growing interest in biological control methods to ensure safe production (Compant et al., 2005; Collinge et al., 2022).

Biological control based on bacteriophages is gaining increasing attention as a means to reduce dependence on chemical pesticides and limit the development of pathogen resistance to antimicrobials (Balogh et al., 2010, Chae et al., 2014). Recently, in Viet Nam, bacteriophages have been studied and demonstrated as a promising agent for controlling bacterial plant pathogens (Van

Truong et al., 2016; Nga et al., 2021). Therefore, the study titled "Bacteriophage efficacy in controlling bacterial leaf spot disease on water spinach (*Ipomoea aquatica*)" was conducted to identify bacteriophage strains that can potentially be used to control bacterial leaf spot disease on water spinach. The research aimed to compare the efficacy of disease prevention and control. The ultimate goal was to determine the most effective bacteriophage-based treatments to mitigate crop losses caused by the disease in water spinach cultivation.

2. MATERIALS AND METHODS

2.1. Isolation, evaluation of pathogenicity and identification of bacterial pathogen causing leaf spot on spinach

2.1.1. Isolation of bacterial pathogens

Samples were collected from February to September 2019 from water spinach fields exhibiting symptoms of bacterial leaf spot disease in Cho Moi district (An Giang province) and Binh Tan district (Vinh Long province) within the Mekong Delta region.

For isolation of causal bacteria of leaf spot on water spinach, a small section (~3 mm) of leaf tissue was excised from the interface between healthy and diseased tissue and finely chopped. The sample was placed in 1–2 drops of sterile distilled water and left for one minute to allow bacterial streaming. The preparation was then observed under a light microscope using a 10X objective lens to confirm the presence of bacterial exudates. One drop of the bacterial suspension was subsequently streaked on King's B medium with 2% agar and incubated at room temperature (28 ± 2°C) for 48 hours to isolate bacterial colonies. Both CM and BT strains were tested for pathogenicity. Each bacterial strain was tested for whether it caused a hypersensitive reaction after 24 hours of filtration with bacterial suspension (OD 600nm: 0.3) into chili pepper leaves (*Capsicum annuum*) to determine whether it belonged to a plant bacterial pathogen or not. The strains were also tested in accordance with Koch's postulates by inoculating healthy water spinach plants. Typical bacterial leaf spot symptoms appeared within 48 hours post-inoculation in both cases. The bacteria were then re-isolated from symptomatic leaves and confirmed to be identical to the original isolates, thus fulfilling Koch's postulates. Two purified bacterial isolates, i.e., strain CM from Cho Moi and strain BT from Binh Tan, exhibited yellow pigmentation characteristic of *Xanthomonas* spp. and were preserved on King's B agar slants at 4 °C. These strains were used in pathogenicity assays and in evaluating bacteriophage efficacy.

2.1.2. Evaluation of the pathogenicity of two bacterial strains causing bacterial leaf spot disease on water spinach under net house conditions

The purpose of the experiment is to evaluate the pathogenicity of two bacterial strains isolated from the previous experiment that cause bacterial leaf spot disease in water spinach.

The experiment followed a completely randomized design with three replications and two treatments, corresponding to strains CM and BT.

Thuan Dien variety of water spinach was sown in plastic bags containing 1.5 kg of soil. Seeds were treated in warm water before sowing. Plants were cared for and fertilised adequately with nitrogen at 5g/litre of water, and fertilised at 5, 10, and 15 days after sowing.

The pathogenic bacteria were cultured on King'B medium for 48 hours at room temperature (28 ± 2°C), then diluted to $OD_{600\ nm}$ = 0.3 (corresponding to a bacterial density of $5x10^7$ cfu/ml).

Inoculation was performed when water spinach plants were 18 days old by spraying 30 ml of bacterial suspension (OD 600nm: 0.3) onto all leaves. Plant pots were covered with plastic bags and were put in a growth chamber at 25 °C in darkness for 24 hours before transferring to net house. Disease incidence and percentage of diseased leaf area were evaluated when obvious symptoms occurred after inoculation.

Recorded parameters: Disease incidence (%) = (total number of diseased leaves x 100) / total number of observed leaves.

Percentage of diseased leaf area: objectively estimated by visual assessment according to Lang et al. (2007) on all diseased leaves.

2.1.3. Identification of bacteria causing bacterial leaf spot disease on water spinach

The bacteria causing bacterial leaf spot disease in water spinach (CM strain selected from experiment 2) were identified using two methods. The biochemical method was carried out following the protocol described by Schaad et al. (2001), and included Gram testing with 3% KOH (Shurtleff &

Averre, 1997), assessment of carbohydrate degradation and oxidation ability (Hanson, 2008), fluorescence testing under UV light (365 nm), and culture on YDC medium. The molecular identification of the bacterial isolate was performed by amplifying the 16S rDNA using universal primers 27F and 1492R (Zhang et al., 2020). PCR reaction was performed according to the thermal cycle: initial denaturation at 94^0C for 4 minutes, followed by 35 cycles (94°C for 45 seconds, 55°C for 45 seconds and 72°C for 90 seconds), finally 72°C for 10 minutes and held at 4°C. PCR products were electrophoresed on 1% agarose gel in 1X TAE buffer at 50V for 30 minutes, and PCR products were sent for sequencing by Phu Sa Biotech Co., Ltd. The resulting sequence was compared with reference sequences in the NCBI 16S rRNA database using the BLAST tool (http://ncbi.nlm.nih.gov/BLAST) (Altschul et al., 1990). The bacterial genus was identified based on 16S rDNA sequence similarity.

2.2. Isolation and screening promising bacteriophages for controlling bacterial leaf spot on water spinach

The objective is to select bacteriophages with high inhibitory activity against bacterial pathogens *in vitro* and high effects against leaf spot disease on water spinach under net house and field conditions.

2.2.1. Isolation of bacteriophages

For bacteriophage isolation, the method of Giang and Nga (2016) was followed. Samples included diseased leaf tissues, roots, and adhering rhizospheric soil collected from the same water spinach plants. Each sample type (leaf or root with soil) was ground separately in a sterile mortar using 5 mL of sterile distilled water, then centrifuged in falcon tubes at 6000 rpm for 5 minutes. After centrifugation, 1 mL of the clear supernatant containing bacteriophages was extracted. A 50 µL volume of chloroform was added, mixed well, and left for 5 minutes. The mixture was centrifuged again at 6000 rpm for 5 minutes to remove bacteria, finally obtaining a clear solution containing only bacteriophages. The clear solution was combined with a suspension of bacterial pathogen in King's B medium with melted 0.8% agar at 50°C. The plates were incubated at room temperature, and the formation of plaques on the bacterial lawn was monitored.

The bacteriophages isolated from this experiment were used for subsequent studies to evaluate the effectiveness of controlling bacterial leaf spot disease on water spinach.

2.2.2. Evaluation of bacteriophage in lysis Xanthomonas sp. causing leaf spot on water spinach under laboratory conditions

The study aims to identify bacteriophages with high lytic activity against *Xanthomonas* sp., the causal agent of leaf spot on water spinach.

The experiment was arranged in a completely randomized design with three replications, using five bacteriophages (Φ3a, Φ5b, Φ7a, Φ8b, Φ9) and one highly virulent bacterial strain (CM). After propagation of each bacteriophage on soft King B medium 0.8% agar, the phage was harvested before determining its titer. The phage suspensions were then diluted to 10^9 pfu/ml using sterile distilled water, which was used in further experiments. After cultures of *Xanthomonas* sp. CM strains on King's B medium with 2% agar for 24 hours, bacterial suspension with OD_{600nm} of 0.3 were prepared using sterile distilled water. The tests of lysis ability of different phages were conducted by combined bacteriophage suspension of each phage with the CM bacterial strain suspension directly in Petri dishes containing 10 ml King's B medium with 0.8% agar at 50°C. These plates were incubated at room temperature for 48 hours. Among the twelve bacteriophages isolated from diseased leaves and rhizospheric soil, five phages were selected for further experiments. The selection was based on the relative size of lysis plaques produced on bacterial lawns during initial screening with *Xanthomonas* sp. strain CM, which is commonly considered a preliminary indicator of lytic potential and host range (Gu et al., 2012).

Plaque diameters formed by each bacteriophage lysis on bacterial lawn of *Xanthomonas* sp. strain CM were observed and recorded by measuring the diameter of ten plaques per replication at 24, 36, and 48 hours after inoculation.

2.2.3. Evaluation of bacteriophage efficacy in controlling bacterial leaf spot disease on water spinach under net house conditions

This study evaluates the effectiveness of two promising bacteriophages, Φ8b and Φ9, against the virulent pathogen *Xanthomonas* sp. strain CM to control bacterial leaf spot disease on water spinach under net house conditions.

The experiment was arranged in a randomized complete block design with one factor, including five treatments and four replications.

- Treatment 1: Control (untreated).
- Treatment 2: Φ8b treatment (10^8 pfu/ml).
- Treatment 3: Φ9b treatment (10^8 pfu/ml).
- Treatment 4: Mixed bacteriophage treatment including: Φ8b and Φ9b (10^8 pfu/ml).
- Treatment 5: Chemical treatment with Starner 20WP (Oxolinic acid 20% w/w; Sumitomo Chemical Co., Ltd.,).

The water spinach pot experiment was conducted as described above.

Three phage treatments were sprayed with bacteriophage suspension (Φ8b, Φ9b, and mixed bacteriophages, respectively) at 10^8 pfu/ml onto leaves of plant 18 days after sowing, and in the sunset, a control treatment was sprayed with an equal volume of water. Subsequent *Xanthomonas* sp. strain CM suspensions were sprayed on leaf surface for all treatments.

Starner 20 WP was applied as a foliar spray at the recommended dosage when disease incidence reached approximately 10% in the chemical treatment.

The total number of diseased leaves and the total number of leaves within each frame were counted at 7, 8, and 9 days after inoculation (DAI).

Disease incidence (%) = (total number of diseased leaves x 100) / total number of observed leaves

The percentage of diseased leaf area was calculated as described above.

The area under the disease progress curve (AUDPC) was calculated using the formula described by Simko and Piepho (2012).

$$AUDPC = \sum_{i=1}^{n=1} \left(\frac{y_i + y_{i+1}}{2}\right)(t_{i+1} - t_i)$$

Where: n = number of assessments.

y_i = disease incidence or percentage of diseased leaf area at the i^{th} observation.

t_i = time of the i^{th} observation.

2.2.4. Evaluation of bacteriophage efficacy in controlling bacterial leaf spot disease on water spinach under field conditions

The objective of this study was to select bacteriophages with high inhibitory efficacy against leaf spot disease on water spinach under field conditions

The study was conducted under field conditions at the Agricultural Experimental Farm, College of Agriculture, Can Tho, from August to September 2019.

The field experiment was prepared on an area covering 121 m², which consisted of 20 plots, each measuring 1 m × 4 m (4 m²). The plots were separated by 0.5 m buffer zones to prevent cross-contamination. Cultural practices, including sowing density, fertilization, and pest and fungal disease control, were applied based on farmer surveys. No chemical bactericides were used in the experimental field. The fertilization schedule during cultivation was implemented according to Table 1.

Table 1. Fertilization schedule applied to water spinach in field experiment

Timing	Fertilizer Amount
Basal fertilization	Lime powder 30g/m², organic microbial fertilizer 100g/m²
5 days after sowing	20-20-15: 3g/m²
10 days after sowing	20-20-15: 5g/m²
15 days after sowing	20-20-15: 5g/m²

Methods for bacteriophage application, pathogen inoculation, and Starner 20WP treatment were similar to those in experiment 2.2.3.

Data collection was conducted by placing five frames, each measuring 400 cm² (20 cm × 20 cm), diagonally on every plot at a distance of 0.5 m from the plot edge. Within each frame, disease incidence and the percentage of diseased leaf area were recorded at 5, 6, and 7 days post-inoculation, following the methodology described in experiment 2.2.3.

Statistical analysis was carried out by processing the data using Microsoft Excel, while further statistical evaluation was performed with the MstatC software. Treatment means were compared using Duncan's Multiple Range Test at a 5% significance level ($p < 0.05$). For experiments involving only two treatments, comparisons were conducted using the T-test

3. RESULTS AND DISCUSSION

3.1. Isolation, pathogenicity and identification of bacterial pathogens causing leaf spot on water spinach

3.1.1. Result of isolation of bacterial pathogen

Two bacterial strains were successfully isolated from water spinach leaf samples with leaf spot

symptoms from Cho Moi - An Giang (CM strain) and Binh Tan - Vinh Long (BT strain). The bacterial colonies were shiny yellow. Disease symptoms began with light green spots that turned dark brown with yellow halos, 2-3 mm in diameter, spreading and coalescing. Older leaves turned yellow, with the disease developing strongly on lower leaves. The colony characteristics and disease symptoms are consistent with previous descriptions by (Leksomboon & Thaveechai, 1991).

3.1.2. Result of evaluation of the pathogenicity of two bacterial strains causing bacterial leaf spot disease on water spinach under net house conditions

Based on the results in Table 2, the disease incidence in the treatment with the bacterial strain CM was consistently higher and significantly different from that in the treatment with the bacterial strain BT across all observation periods.

Table 2. Disease incidence at different time points

Treatment	Disease Incidence (%)		
	7 DAI	8 DAI	9 DAI
strain CM	74.29[a]	77.02[a]	78.98[a]
strain BT	47.28[b]	53.59[b]	56.85[b]
P value	0.0285	0.0111	0.0072

In the same column, mean values followed by the same letter are not significantly different according to the T-test. DAI: days after inoculation.

At 7 days after inoculation (DAI), the strain CM demonstrated a notably higher pathogenicity with an incidence rate of 74.29% compared to 47.28% for the strain BT.

At 8 DAI, although the disease progression from day 7 was lower than in BT (2.92% versus 6.31%), the overall disease incidence of CM remained significantly higher (77.02% versus 53.59%).

By 9 DAI, both strains showed a slowdown in disease development, with leaf yellowing and dropping that hindered disease spread. In nature plant defense that plants can initiate both systemic and localized immune responses against bacterial pathogens. For instance, rice infected with *Xanthomonas oryzae* pv. *oryzae,* the upregulation of pathogenesis-related (PR) genes and accumulation of reactive oxygen species (ROS) contribute to a hypersensitive-like defense response that restricts bacterial proliferation (Dai *et al.*, 2023). While the defense mechanisms in water spinach (*Ipomoea aquatica*) remain uncharacterized, the disease pattern observed in this study aligns with plant immune responses known to be effective against *Xanthomonas* infections. However, strain CM still maintained a higher disease incidence (78.98% compared to 56.85% for BT) (Figure 1).

Figure 1. Level of leaf spot infection on water spinach plants caused by bacterial strains (a. strain CM, b. strain BT) at 9 DAI after inoculation

Regarding the percentage of diseased leaf area caused by two bacterial strains on water spinach, as shown in Table 3, the result indicated that the bacterial strain CM consistently presented with a higher percentage of diseased leaf area.

Table 3. Percentage of diseased leaf area at different time points when inoculated with two bacterial strains causing leaf spot on water spinach

Treatment	Percentage of diseased leaf area (%)		
	7 DAI	8 DAI	9 DAI
strain CM	56.19[a]	67.03[a]	67.09[a]
strain BT	18.87[b]	24.40[b]	29.11[b]
P value	0.0072	0.0029	0.0151

In the same column, mean values followed by the same letter are not significantly different according to the T-test. DAI: days after inoculation.

It is concluded that the bacterial strain CM has a stronger pathogenicity than the strain BT. Therefore, strain CM was selected as the source of the pathogen for subsequent experiments in identification and phage study.

3.1.3. Result of identification of bacterial strain causing bacterial leaf spot disease in water spinach

Identification based on biochemical characteristics described by Schaad et al. (2001) determined that the bacteria were Gram-negative, aerobic, non-fluorescent on King's B medium, and formed yellow colonies on YDC medium, thereby preliminarily concluding that the bacteria belonged to either the genus Xylophilus or Xanthomonas. Following amplification of the 16S rDNA segment (approximately 1500 bp) and Sanger sequencing, the sequence was classified within the genus Xanthomonas, consistent with the previous study by Leksomboon and Thaveechai (1991).

3.2. Result of the isolation and screening promising bacteriophages for controlling bacterial leaf spot on water spinach

3.2.1. Result of bacteriophage isolation

Twelve bacteriophages were isolated from twelve initial samples, including seven leaf samples and five soil samples (Table 4). The presence of bacteriophages in both soil and diseased leaves is similar to that described in previous studies on *Erwinia amylovora* (Gill & Abedon, 2003). Bacteriophages infect phylosphere bacterial pathogens, which can survive both on infected foliar tissue and also soil, as water washes them to the soil. In soil which can penetrate the plant canopy during seed germination and move between plants in the environment (Gill & Abedon, 2003). The plaque diameters of phages ranged from 4.25 to 5.8 mm. Five bacteriophages with the largest plaque diameter (Φ3a, Φ5b, Φ7a, Φ8b, Φ9) were selected for further study.

Table 4. List of isolated bacteriophages of *Xanthomonas* causing bacterial leaf spot on water spinach

Phage code	Isolation Location	Orginal isolation	plaque diameter (mm)
Φ1a	Binh Tan, Vinh Long	leaf	4.3
Φ1b	Binh Tan, Vinh Long	leaf	3.6
Φ2	Phu Tan, An Giang	leaf	4.8
Φ3a	Phu Tan, An Giang	soil	5.6
Φ3b	Phu Tan, An Giang	soil	4.3
Φ5a	Cho Moi, An Giang	leaf	4.5
Φ5b	Cho Moi, An Giang	leaf	5.2
Φ7a	Long Xuyen, An Giang	leaf	5.4
Φ7b	Long Xuyen, An Giang	leaf	4.6
Φ8a	PhuTan, An Giang	soil	4.3
Φ8b	Phu Tan, An Giang	soil	5.8
Φ9	Thot Not, Can Tho	soil	5.3

3.2.2. Lysis capability of bacteriophages against Xanthomonas sp. causing leaf spot disease under laboratory conditions

The results of evaluating the lysis capability of five bacteriophages (Φ3a, Φ5b, Φ7a, Φ8b, Φ9) against *Xanthomonas* sp. strain CM based on plaque diameter at three time points: 24, 36, and 48 hours are with the average plaque diameter from 5.07 to 11.73 mm during three time courses of observation (Table 5, Figure 2).

Notably, two bacteriophages Φ8b and Φ9 exhibited superior lysis capability and significant differences compared to the other three phages at all time points. At 24 hours, Φ9 and Φ8b had the largest diameters (6.92 mm and 5.80 mm, respectively). By 36 hours, all phages increased plaque diameter, with Φ9 showing the largest increase (up to 10.29 mm). At 48 hours, Φ9 continued to lead, with a plaque diameter of 11.73 mm, which was significantly different from those of the other phages. Φ8b ranked second with 6.53 mm, while the remaining three phages had smaller plaque diameters, ranging from 5.16 to 5.57 mm. Plaque diameter was used as a preliminary indicator of lytic capacity, where larger plaques reflect stronger bacterial clearance and faster diffusion through the bacterial lawn. This criterion is consistent with previous findings (Kutter & Sulakvelidze, 2005). Hence, Φ9 and Φ8b were selected for subsequent net house trials.

Table 5. Plaque diameter of five bacteriophages at different time points

Treatment	plaque diameter (mm)		
	24 hours	36 hours	48 hours
Φ3a	5.07^{c}	5.19^{c}	5.57^{c}
Φ5b	4.46^{d}	4.85^{c}	5.16^{c}
Φ7a	4.95^{c}	5.16^{c}	5.31^{c}
Φ8b	5.80^{b}	6.06^{b}	6.53^{b}
Φ9	6.92^{a}	10.29^{a}	11.73^{a}
P value	0.0001	0.0001	0.0001

In the same column, values followed by the same letter are not significantly different according to Duncan's Multiple Range Test,

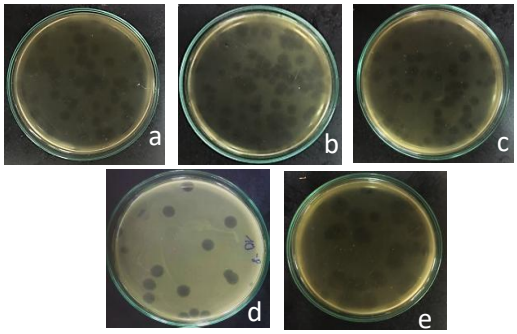

Figure 2: Plaque size of five phages at 48 hours after culturing on soft agar a) Φ3a, b) Φ5b, c) Φ7a, d) Φ8b, e) Φ9

3.2.3. Efficacy of bacteriophage in controlling bacterial leaf spot disease in water spinach under net house conditions

The experiment to assess the application of single bacteriophages Φ8b or Φ9, and a mixture of the two phages in controlling leaf spot on water spinach caused by *Xanthomonas* strain CM under net house conditions. The results are presented in Tables 6, 7, and Figure 3.

Disease incidence results showed that single-bacteriophage treatments were more effective than the mixed-bacteriophage treatment. The chemical treatment (Starner 20WP) was the most effective and stable across all time points (Table 6). At 7 days after inoculation (DAI), two phage treatments, Φ8b (30.69%) and Φ9 (29.33%), exhibited lower disease incidence compared to the control, while the mixed phage treatment did not differ significantly from the control. The Starner treatment consistently showed the lowest disease incidence. At 8 DAI, the Starner treatment again had the lowest disease incidence (19.58%). Treatments with Φ8b (36.70%) and Φ9 (34.92%) demonstrated significant differences compared to the control (53.80%), whereas the mixed phage treatment did not differ significantly from the control. Similar results were observed at 9 DAI.

Table 6. Disease incidence when treated with bacteriophage treatments and Starner 20WP fungicide under net house conditions

Treatment	Disease Incidence (%)			
	7 DAI	8 DAI	9 DAI	AUDPC
Φ8b	30.69^{b}	36.70^{b}	36.30^{b}	113.23^{b}
Φ9	29.33^{b}	34.92^{b}	37.62^{b}	110.08^{b}
Mix	45.04^{a}	50.98^{a}	53.99^{a}	157.48^{a}
Starner 20WP	18.65^{c}	19.58^{c}	20.43^{c}	62.26^{c}
Control	45.91^{a}	53.80^{a}	59.98^{a}	171.61^{a}
P value	0.0001	0.0001	0.0001	0.0001

In the same column, values followed by the same letter are not significantly different according to Duncan's Multiple Range Test, DAI: days after inoculation. Mix: combination of bacteriophages Φ8b and Φ9.

Regarding AUDPC, the chemical treatment was the lowest and significantly different from the other treatments. Both Φ8b and Φ9 differed significantly from the control at the 5% significance level.

Table 7. Percentage of diseased leaf area when treated with bacteriophage treatments and Starner 20WP under net house conditions

Treatment	Percentage of diseased leaf area (%)		
	7 DAI	8 DAI	9 DAI
Φ8b	5.44^{c}	6.09^{c}	6.44^{c}
Φ9	7.06^{bc}	7.33^{bc}	7.66^{bc}
Mix	10.64^{ab}	11.22^{b}	11.29^{b}
Starner 20WP	7.96^{bc}	8.72^{bc}	8.23^{bc}
Control	14.68^{a}	16.75^{a}	17.02^{a}
Significance	0.0089	0.002	0.0017

Note: In the same column, values followed by the same letter are not significantly different according to Duncan's Multiple Range Test at the 5% Mix: combination of bacteriophages Φ8b and Φ9.

The percentage of diseased leaf area differed significantly among all three bacteriophage treatments compared to the control (Table 7). Phage Φ8b exhibited a lower average percentage of diseased leaf area than the mixed phage treatment and was comparable to Φ9 and the Starner treatment. At 7 days after inoculation (DAI), the three treatments differed significantly from the control, with Φ8b showing the lowest percentage of diseased leaf area (5.44%), followed by Φ9 (7.06%)

and the chemical treatment (7.96%). The mixed phage treatment did not differ significantly from the control. At 8 DAI, all four treatments differed significantly from the control, with Φ8b again showing the lowest percentage (6.09%), which was significantly different from the mixed phage treatment (11.22%). Similar results were observed at 9 DAI (Figure 3). Overall, bacteriophages effectively controlled bacterial leaf spot disease caused by *Xanthomonas* sp. in water spinach, consistent with the findings of Balogh et al. (2010), which reported disease reductions of 23–33%.

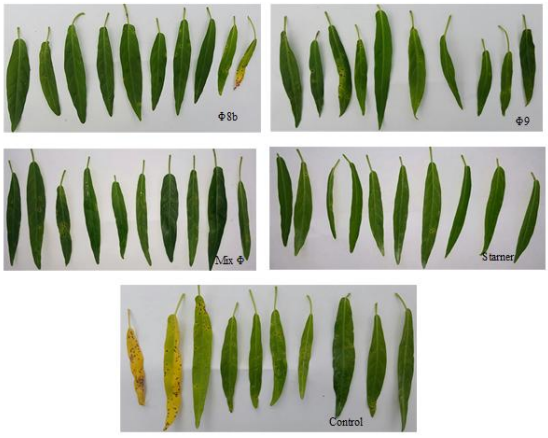

Figure 3. Control efficacy of bacteriophages and Starner 20WP compared to the control at 10 days after inoculation under net house conditions

3.2.4. Effectiveness of bacteriophages in controlling bacterial leaf spot disease of water spinach under field conditions

Disease incidence: treatments with the bacteriophages Φ8b, Φ9, and the Starner 20WP bactericide showed significantly lower disease rates than the control over time (Table 8).

At 5 DAI, treatments with Φ8b, Φ9, and Starner 20WP had disease incidence of 6.9% to 13.1%, significantly lower than the control (23.7%).

At 6 DAI, Φ8b, Φ9, and Starner 20WP showed disease-reduction efficacy, with incidence ranging from 7.4% to 15.2%, significantly lower than the control (25.1%). The mixed bacteriophage treatment (20.2%) showed no significant difference compared to the control.

Similarly, at 7 DAI, Φ8b, Φ9, and Starner 20WP continued to demonstrate disease reduction efficacy. The mixed bacteriophage treatment (25.7%) showed no significant difference compared to the control.

As shown in Table 8, AUDPC values for single-bacteriophage treatments and Starner 20WP were all significantly lower than those of the control. However, AUDPC values for single-bacteriophage treatments were significantly higher than those for the Starner 20WP treatment. The mixed bacteriophage treatment showed no significant difference in AUDPC compared to the control.

Table 8. Disease incidence when treated with bacteriophage treatments and Starner 20WP under field conditions

Treatment	Disease Incidence (%)			AUDPC
	5 DAI	6 DAI	7 DAI	
Φ8b	13.1^{b}	15.6^{b}	18.6^{b}	31.5^{b}
Φ9	12.5^{b}	15.2^{b}	18.2^{b}	30.6^{b}
Mix	17.7^{ab}	20.2^{ab}	25.7^{ab}	41.9^{ab}
Starner 20WP	6.9^{c}	7.4^{c}	7.7^{c}	14.7^{c}
Control	23.7^{a}	25.1^{a}	29.1^{a}	52.0^{a}
P value	0.0001	0.0001	0.0001	0.0001

In the same column, values followed by the same letter are not significantly different according to Duncan's Multiple Range Test, DAI: days after inoculation. Mix: combination of bacteriophages Φ8b and Φ9.

The percentage of diseased leaf area differs among treatments with bacteriophages, chemical treatment, and control across observation times. Treatments with the bacteriophages Φ8b, Φ9, and Starner 20WP demonstrate preventive efficacy, with significantly lower diseased leaf area than the control (Table 9).

Table 9. Percentage of diseased leaf area when treated with bacteriophage treatments and Starner 20WP under field conditions

Treatment	Percentage of diseased leaf area (%)		
	5 DAI	6 DAI	7 DAI
Φ8b	0.48^{bc}	0.66^{b}	0.73^{bc}
Φ9	0.49^{bc}	0.73^{b}	0.96^{b}
Mix	0.67^{ab}	1.01^{ab}	0.12^{ab}
Starner 20WP	0.29^{c}	0.25^{c}	0.34^{c}
Control	1.00^{a}	1.32^{a}	1.47^{a}
P value	0.0004	0.0001	0.0001

In the same column, values followed by the same letter are not significantly different according to Duncan's Multiple Range Test at the 5% significance level, DAI: days after inoculation. Mix: combination of bacteriophages Φ8b and Φ9.

At 5 DAI, Starner 20WP shows the lowest percentage of diseased leaf area (0.29%), followed by Φ8b (0.48%) and Φ9 (0.49%), significantly

lower than the control (1.00%). The Mix treatment shows no significant difference compared to the control.

At 6 and 7 DAI, Starner 20WP remains the most effective treatment in disease reduction, followed by phage Φ8 and Φ9, significantly different from the control. Mix shows no significant difference compared to the control.

Results indicate that Starner 20WP demonstrates the best disease reduction effect, followed by Φ8 and Φ9, while the Mix treatment does not provide significant disease reduction. This phenomenon is likely associated with viral interference, in which co-infection of a host cell by multiple viruses, each replicating independently, can result in the suppression of one virus by another (Cao & Nguyen, 2011). Similar observations were reported by Chan et al. (2013), who found that certain combinations of bacteriophages were less effective than individual applications due to mutual inhibition or competitive dynamics. Therefore, careful evaluation of phage compatibility is essential when formulating cocktails. In this study, bacteriophages Φ8b and Φ9 were selected based on their larger plaque diameters in vitro, and both exhibited consistent and high disease suppression efficacy under both net house and field conditions. This consistency supports the relevance of plaque morphology as a preliminary selection criterion for phage efficacy. Our findings align with previous studies reporting that larger plaques are often associated with higher virulence and more efficient host lysis (Balogh et al., 2010; Gill & Hyman, 2010).

The effectiveness of bacteriophages Φ8b and Φ9 in reducing bacterial leaf spot on water spinach is in line with findings from previous studies on other crops. For example, Balogh et al. (2010) reported successful phage-based suppression of *Xanthomonas euvesicatoria* in tomato and pepper, while Chae *et al.* (2014) demonstrated phage efficacy against *X. campestris* pv. *campestris* on cabbage. These results collectively support the broader application of phage therapy for the management of bacterial diseases in vegetables.

4. CONCLUSION

Bacterial leaf spot disease on water spinach was associated with *Xanthomonas* sp.

Five bacteriophages, i.e., Φ3a, Φ5b, Φ7a, Φ8b, and Φ9, showed the largest plaque diameters in a collection of 12 isolated bacteriophages. In which, phage Φ8b and Φ9 expressed the largest plaque diameters during the time courses of observation.

In the net house, foliar application of phage Φ8b, or Φ9 suspension at a titer of 10^8 pfu/ml, was effective in disease reduction. However, the disease reduction of phage treatment showed lower Starner treatment.

In the field trial, individual treatments of Φ8b and Φ9 were more effective than the mixture (Φ8b + Φ9) and the control. Starner 20WP provided the highest control efficacy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of molecular biology*, *215*(3), 403-410. https://doi.org/10.1016/S0022-2836(05)80360-2

Ansari, M., Moraiet, M., & Ahmad, S. (2014). Insecticides: Impact on the environment and human health. In: Malik, A., Grohmann, E., & Akhtar, R. (Eds.), *Environmental Deterioration and Human Health* (pp. 99-123) Springer, Dordrecht. https://doi.org/10.1007/978-94-007-7890-0_6

Balogh, B., Canteros, B. I., Stall, R. E., & Jones, J. (2008). Control of citrus canker and citrus bacterial spot with bacteriophages. *Plant Disease*, *92*(7), 1048-1052. https://doi.org/10.1094/PDIS-92-7-1048

Balogh, B., Jones, J. B., Iriarte, F., & Momol, M. (2010). Phage therapy for plant disease control. *Current Pharmaceutical Biotechnology*, *11*(1), 48-57. http://dx.doi.org/10.2174/138920110790725302

Cao, N. D., & Nguyen, H. H. (2011). *Veterinary Virology*. Hanoi: Agriculture Publishing House.

Chae, J. C., Nguyen, B. H., Yu, S. M., Lee, H. K., & Lee, Y. H. (2014). Diversity of bacteriophages infecting Xanthomonas oryzae pv. oryzae in paddy fields and its potential to control bacterial leaf blight of rice. *Journal of Microbiology and Biotechnology*, *24*(6), 740-747. https://doi.org/10.4014/jmb.1402.02013

Chan, B. K., Abedon, S. T., & Loc-Carrillo, C. (2013). Phage cocktails and the future of phage therapy. *Future Microbiology*, *8*(6), 769–783. https://doi.org/10.2217/fmb.13.47

Collinge, D. B., Jensen, D. F., Rabiey, M., Sarrocco, S., Shaw, M. W., & Shaw, R. H. (2022). Biological control of plant diseases–What has been achieved and what is the direction? *Plant Pathology*, *71*(5), 1024-1047. https://doi.org/10.1111/ppa.13555

Compant, S., Duffy, B., Nowak, J., Clément, C., & Barka, E. A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Applied and Environmental Microbiology*, *71*(9), 4951-4959. https://doi.org/10.1128/aem.71.9.4951-4959.2005

Chae, Y. H., Choi, S. K., & Lee, S. W. (2014). Isolation and characterization of bacteriophages against Xanthomonas campestris pv. campestris. *Plant Pathology Journal*, *30*(2), 175–180. https://doi.org/10.3390/v14071449

Dasgupta, S., Meisner, C., Wheeler, D., Xuyen, K., & Lam, N. T. (2007). Pesticide poisoning of farm workers–implications of blood test results from Vietnam. *International Journal of Hygiene and Environmental Health*, *210*(2), 121-132. https://doi.org/10.1016/j.ijheh.2006.08.006

Dai, X., Wang, Y., Yu, K., Zhao, Y., Xiong, L., Wang, R., & Li, S. (2023). OsNPR1 enhances rice resistance to *Xanthomonas oryzae* pv. *oryzae* by upregulating rice defense genes and repressing bacteria virulence genes. *International Journal of Molecular Sciences*, *24*(10), 8687. https://doi.org/10.3390/ijms24108687

Duc, B. M., Humphries, D., Mai, L. T. B., Dao, H. A., Co, T. M., Nga, H. H., & Kim, P. T. (1999). Iron and vitamin C content of commonly consumed foods in Vietnam. *Asia Pacific Journal of Clinical Nutrition*, *8*(1), 36-38. https://doi.org/10.3390/ijms24108687

Giang, N. T. T., & Nga, N. T. T. (2016). Survey of methods for isolating bacteriophages and the effectiveness of bacteriophages in preventing and treating bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae*. Proceedings of the 15th National Conference on Plant Diseases in Vietnam (pp. 30-39).

Gill, J., & Abedon, S. T. (2003). *Bacteriophage ecology and plants*. APS net.

Gill, J. J., & Hyman, P. (2010). Phage choice, isolation, and preparation for phage therapy. *Current Pharmaceutical Biotechnology*, *11*(1), 2–14. http://dx.doi.org/10.2174/138920110790725311

Gu, J., Liu, X., Li, Y., Han, W., Lei, L., Yang, Y., Zhao, H., Gao, Y., Song, J., Lu, R., Sun, C., & Feng, X. (2012). A method for generation phage cocktail with great therapeutic potential. *PLoS One*, *7*(3), e31698. https://doi.org/10.1371/journal.pone.0031698

Hanson, R. S. (2008). Methanotrophic bacteria. Microbiological Reviews, 60(2), 439-471. https://doi.org/10.1128/mr.60.2.439-471.1996

Kutter, E., & Sulakvelidze, A. (2005). *Bacteriophages: Biology and applications*. CRC Press.

Lang, J. M., Gent, D. H., & Schwartz, H. F. (2007). Management of Xanthomonas leaf blight of onion with bacteriophages and a plant activator. *Plant Disease*, *91*(7), 871-878. https://doi.org/10.1094/PDIS-91-7-0871

Leksomboon, C. & Thaveechai, N. (1991). Bacterial leaf spot of Chinese convolvulus [Ipomoea aquatica]. In *Proceedings of the 29th Kasetsart University: Plant Science*, (Bangkok: Kasetsart University), (pp. 277-283).

Marcussen, H., Joergensen, K., Holm, P. E., Brocca, D., Simmons, R. W., & Dalsgaard, A. (2008). Element contents and food safety of water spinach (Ipomoea aquatica Forssk.) cultivated with wastewater in Hanoi, Vietnam. *Environmental Monitoring and Assessment*, *139*, 77-91. https://doi.org/10.1094/PDIS-03-22-0689-PDN

Nga, N. T. T, Tran T. N., Holtappels D., Kim Ngan, N. L., Hao, N. P., Vallino M., Tien, D. T. K., Khanh-Pham, N. H., Lavigne, R., Kamei, K., Wagemans, J., & Jones, J. B. (2021). Phage biocontrol of bacterial leaf blight disease on welsh onion caused by Xanthomonas axonopodis pv. allii. *Antibiotics*, *10*(5), 517. https://doi.org/10.3390/antibiotics10050517

Schaad, N. W., Jones, J. B., & Chun, W. (2001). *Laboratory guide for identification of plant pathogenic bacteria* (3rd ed.). St. Paul, MN: APS Press.

Shurtleff, M. C., & Averre, C. W. (1997). *The plant disease clinic and field diagnosis of abiotic diseases*. American Phytopathological Society.

Simko, I., & Piepho, H. P. (2012). The area under the disease progress stairs: Calculation, advantage, and application. *Phytopathology*, *102*(4), 381-389. https://doi.org/10.1094/PHYTO-07-11-0216

Van Truong, T., B., Pham Khanh, N. H., Namikawa, R., Miki, K., Kondo, A., Dang Thi, P. T., & Kamei, K. (2016). Genomic characterization of Ralstonia solanacearum phage ϕRS138 of the family Siphoviridae. *Archives of Virology*, *161*(2), 483–486. https://doi.org/10.1007/s00705-015-2654-1

Zhang, W., Chen, Y., Shi, Q., Hou, B., & Yang, Q. (2020). Identification of bacteria associated with periapical abscesses of primary teeth by sequence analysis of 16S rDNA clone libraries. *Microbial Pathogenesis*, *141*, 103954. https://doi.org/10.1016/j.micpath.2019.103954