EFFICACY OF BACTERIOPHAGES FOR CONTROLLING *Pectobacterium carotovorum* CAUSING SOFT ROT DISEASE IN CRUCIFEROUS VEGETABLES

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ABSTRACT

Soft rot disease, caused by the bacterium *Pectobacterium carotovorum*, is considered one of the most serious pathogens affecting Cruciferous vegetables, inflicting significant damage during both the cultivation phase and post-harvest. The study on the isolation and selection of bacteriophage capable of controlling soft rot disease in Cruciferous vegetables caused by *P. carotovorum* was conducted under *in vitro* and greenhouse conditions. A total of twenty bacteriophages were isolated from soil and leaf samples collected from three provinces (Can Tho, Vinh Long and Tra Vinh) that were capable of infecting *P. carotovorum*. Among them, eight phages (Φ1, Φ2, Φ3, Φ5, Φ6, Φ15, Φ17, and Φ20) demonstrated a 100% infection rate against the eight tested bacterial strains. Under *in vitro* conditions, four phages (Φ1, Φ3, Φ5, and Φ20) showed high ability to multiply and lyse *P. carotovorum* among the eight phages evaluated. In greenhouse trial, assessing their ability to control soft rot in Bok Choy, phage Φ20 exhibited disease suppression efficacy nearly equivalent to the bactericide Starner 20WP. These findings confirm the potential of bacteriophages as a promising biocontrol agent against soft rot caused by *P. carotovorum* in Cruciferous vegetables.

Keywords: Bacteriophages, Cruciferous vegetables, *Pectobacterium carotovorum*, Soft rot disease

ĐÁNH GIÁ HIỆU QUẢ CỦA THỰC KHUẨN THỂ TRONG PHÒNG TRỊ VI KHUẨN *Pectobacterium carotovorum* GÂY BỆNH THỐI NHŨN TRÊN RAU HỌ CẢI

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TÓM TẮT

Bệnh thối nhũn, do vi khuẩn *Pectobacterium carotovorum* gây ra, được xem là một trong những tác nhân gây hại nghiêm trọng nhất đối với các loại rau họ Cải, gây tổn thất đáng kể cả trong giai đoạn canh tác lẫn sau thu hoạch. Nghiên cứu phân lập và tuyển chọn thực khuẩn thể có khả năng kiểm soát bệnh thối nhũn trên rau họ Cải do *P. carotovorum* gây ra được tiến hành trong điều kiện in vitro và nhà lưới. Tổng cộng hai mươi thực khuẩn thể đã được phân lập từ mẫu đất và lá thu thập tại ba tỉnh Cần Thơ, Vĩnh Long và Trà Vinh, có khả năng ký sinh trên *P. carotovorum*. Trong số đó, tám thực khuẩn thể (Φ1, Φ2, Φ3, Φ5, Φ6, Φ15, Φ17 và Φ20) thể hiện tỷ lệ ký sinh 100% đối với tám chủng vi khuẩn được thử nghiệm. Trong điều kiện in vitro, bốn thực khuẩn thể (Φ1, Φ3, Φ5 và Φ20) cho thấy khả năng nhân lên và tiêu diệt *P. carotovorum* mạnh trong số tám thực khuẩn thể được đánh giá. Trong thí nghiệm nhà lưới, khả năng kiểm soát bệnh thối nhũn trên cải thìa của thực khuẩn thể Φ20 thể hiện hiệu quả ức chế bệnh gần tương đương với thuốc trừ vi khuẩn Starner 20WP. Những kết quả này khẳng định tiềm năng của thực khuẩn thể như một tác nhân sinh học đầy hứa hẹn trong việc kiểm soát bệnh thối nhũn do *P. carotovorum* gây ra trên rau họ Cải.

Từ khóa: Bệnh thối nhũn, *Pectobacterium carotovorum*, Rau họ Cải, Thực khuẩn thể

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1. INTRODUCTION

Cruciferous plants (also known as Brassicaceae or Cruciferae**)** play an important role in agriculture, human health, and the environment (Ba & Thuy, 2019). However, both yield and quality of these vegetables are compromised by diseases, among which soft rot caused by the bacterium *Pectobacterium carotovorum* is considered a primary pathogen, inflicting severe losses during cultivation and postharvest stages (Garge & Nerurkar, 2017). Although chemical bactericides and antibiotics are widely used, the emergence of resistant bacterial strains and the negative impacts on the environment and human health have driven the demand for alternative control agents (Vu & Oh, 2020). In this context, bacteriophages—viruses that parasitize and quickly lyse bacteria—have been applied as biological control agents (Kutter & Sulakvelidze, 2005). For instance, Elhalag et al. (2024) reported that the phages PcaP1EGY and PcaP2EGY exhibit significant biocontrol efficacy against potato soft rot disease caused by *Pectobacterium carotovorum*, both under field conditions and during postharvest storage. Wang et al. (2022) demonstrated that phage vB_RsoP_BMB50, owing to its broad lytic spectrum, high thermal stability, and strong pH tolerance, represents a promising candidate for biocontrol of bacterial wilt disease caused by *Ralstonia solanacearum*. Therefore, the study "Efficacy of bacteriophages for controlling *Pectobacterium carotovorum* causing soft rot disease in cruciferous vegetables" aims to screen promising phage isolates for parasitism and suppression of soft rot in greenhouse, contributing to the development of safe, rotational, or substitute treatments for chemical control in vegetable production.

2. MATERIALS AND METHODS

2.1. Phage isolation and host ranges testing

Eight *Pectobacterium carotovorum* strains isolated from cruciferous vegetable plants were provided by the Department of Plant Protection, College of Agriculture, Can Tho University.

Phage isolation (Nga & Giang, 2016): Soil or diseased leaf tissue samples were chopped and crushed using a mortar and pestle. The homogenized leaves were mixed with an equal volume of sterile distilled water, followed by centrifugation at 6000rpm for 5 minutes. The supernatant was transferred and treated with chloroform at a concentration of 5% and incubated for 5 min, subsequence tubes were centrifuged at 6000rpm for 5 min, then the phage supernatant was transferred to new tubes, the phage suspension was serially diluted tenfold by then isolating by mixing 100µL of each dilution with 10mL of 0.8% King's B soft agar containing a bacterial suspension (the bacterial strain isolated from the same leaf sample) and pouring it on an agar plate. After 24 h incubation, individual plaques with different distinct morphologies were picked up with a sterile toothpick and streaked on a fresh bacterial lawn with a cotton swab. The purified phage after removal of host bacteria was harvested in water then stored at 4°C.

Host range determination of phages: 100µL suspension of each *P. carotovorum* strain (OD_{600} = 0.3) to 10mL King's B agar (0,8%, 50°C), poured onto a Petri dish. Ten microliters of each purified phage preparation were then spotted onto designated sectors of the agar surface. Plates were incubated at room temperature for 24h, and zones of bacterial clearance were recorded as evidence of successful infection.

2.2. Evaluation of bacteriophage lytic and degradative activity against *Pectobacterium carotovorum* on cruciferous vegetables

The experiment was arranged in a completely randomized design with seven treatments (four monophage treatments, one treatment with a cocktail of four phages, a treatment with Starner 20WP and a control treatment without phage application). Each treatment was applied with four replications.

For the lytic–degradation assay, tenfold serial dilutions of each standardized phage suspension were prepared at 10^{-5}, 10^{-6}, and 10^{-7}. In each assay, 100µL of *P. carotovorum* Pc2 suspension (OD_{600} = 0.3) was mixed with 100µL of the appropriate phage dilution. This mixture was then combined with 10mL of molten King's B soft agar (0.8%), poured into a Petri dish, and gently swirled to distribute evenly. Plates were incubated at room temperature, and plaque formation was observed and recorded at 24, 48, and 72 hours post-inoculation.

Data recording: (1) Phage titer (pfu/mL), calculated from plaque counts multiplied by the dilution factor; (2) Plaque diameter (mm), determined by measuring ten randomly selected plaques per plate and calculating the mean at 24h, 48h, and 72h post inoculation.

2.3. Evaluation of the efficacy of phage treatment in greenhouse conditions

The greenhouse experiment was arranged as a completely randomized design with seven treatments (i.e. four monophage treatments, one treatment with a cocktail of four phages, a control treatment without phage application and a treatment with Starner 20WP). Each treatment was replicated three times.

Phage treatments were applied by uniformly spraying 25mL of each phage suspension onto the foliage of each pot at two hours before pathogen inoculation. The bactericide treatment was similarly sprayed with 25mL Starner 20WP solution at recommended dose at the same time point, the control treatment was sprayed with 25mL of sterile distilled water. Pathogen inoculation was sprayed with 25mL of a *Pectobacterium carotovorum* suspension (10^8 CFU/mL) onto each plant to ensure consistent infection pressure across all experimental units.

Disease assessment: Plants were monitored daily for symptom development. When wilting symptoms first appeared, the following parameters were recorded:

- Disease incidence (%) = (Number of diseased plants × 100) / Total number of plants)

- Area Under Disease Progress Curve (Shanner & Finney, 1977):

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

Where,

y_i = disease index at time t_i

y_{i+1} = disease index at time t_{i+1}

t_i: time at which disease index Y_i was recorded

t_{i+1}: time at which disease index Y_{i+1} was recorded

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3. RESULTS AND DISCUSSION

3.1. Isolation of bacteriophages infecting *Pectobacterium carotovorum* causing soft rot in cruciferous vegetables in the Mekong Delta, Vietnam

Twenty bacteriophages were isolated from three provinces in the Mekong Delta - Can Tho, Vinh Long and Tra Vinh - including twelve phages from leaf samples and eight from soil samples (Table 1). According to Gill and Abedon (2003), based on studies of bacteriophages infecting *Erwinia amylovora*, phages are primarily dispersed between plants through foliar contact. The soil served merely as a reservoir for phages originating from the plant canopy rather than acting as a true ecological niche.

Table 1. List of bacteriophages isolated from Mekong Delta provinces

Phage	Host plant	Sample type	Collection site (Mekong Delta, Vietnam)
Φ1	*Brassica integrifolia*	Leaf	Phu Can, Tieu Can, Tra Vinh Province
Φ2	*Brassica integrifolia*		
Φ3	*Brassica juncea*		
Φ4	*Brassica juncea*	Soil	
Φ5	*Brassica juncea*	Leaf	
Φ6	*Brassica juncea*	Soil	
Φ7	*Brassica juncea*	Soil	
Φ8	*Brassica juncea*	Soil	
Φ9	*Brassica rapa* subsp. *chinensis*	Leaf	Trung nhut, Thot Not, Can Tho Province
Φ10	*Brassica rapa* subsp. *chinensis*	Leaf	
Φ11	*Brassica rapa* subsp. *chinensis*	Soil	
Φ12	*Brassica rapa* subsp. *chinensis*	Leaf	
Φ13	*Brassica rapa* subsp. *chinensis*	Leaf	
Φ14	*Brassica rapa* subsp. *chinensis*	Leaf	
Φ15	*Brassica pekinensis*	Leaf	
Φ16	*Brassica pekinensis*	Soil	
Φ17	*Brassica juncea*	Soil	Hung Thanh, Cai Rang, Can Tho Province
Φ18	*Brassica juncea*	Soil	
Φ19	*Brassica juncea*	Leaf	
Φ20	*Brassica juncea*	Leaf	

3.2. Infectivity of bacteriophages against selected *Pectobacterium carotovorum* strains causing soft rot in cruciferous vegetables

The results of testing the parasitic activity of 20 phages against eight selected *P. carotovorum* strains in vitro revealed that four bacterial strains Pc1, Pc2, Pc6, and Pc7 were fully susceptible to all 20 phages, showing a 100% infection rate. Eight phages (Φ1, Φ2, Φ3, Φ5, Φ6, Φ15, Φ17, and Φ20) demonstrated parasitic ability against all eight *P. carotovorum* strains tested. The remaining phages could parasitize a lower number of *P. carotovorum* strains. The data also showed that a single phage could infect one or multiple bacterial strains from different locations. Conversely, a single bacterial strain could be lysed by several different

phages. This observation supports the findings of Tanaka et al. (1990), who reported that bacteriophages exhibit high specificity replicating only in one or a few host strains of the same species by disrupting DNA, RNA, and protein synthesis, ultimately leading to host-cell lysis. Such specificity is beneficial because it avoids harming non-target beneficial bacteria, although it also limits the range of hosts that can be biocontrolled.

Table 2. Evaluation of the host spectrum of twenty *Pectobacterium carotovorum* phage isolates against eight *Pectobacterium carotovorum* strains

Phages	*Pectobacterium carotovorum* strains								Total
	Pc1	Pc2	Pc3	Pc4	Pc5	Pc6	Pc7	Pc8	
Φ1	+	+	+	+	+	+	+	+	8
Φ2	+	+	+	+	+	+	+	+	8
Φ3	+	+	+	+	+	+	+	+	8
Φ4	+	+	+	-	-	+	+	-	5
Φ5	+	+	+	+	+	+	+	+	8
Φ6	+	+	+	+	+	+	+	+	8
Φ7	+	+	-	-	+	+	+	+	6
Φ8	+	+	+	-	-	+	+	+	6
Φ9	+	+	-	-	-	+	+	+	5
Φ10	+	+	-	+	-	+	+	+	6
Φ11	+	+	-	+	-	+	+	+	6
Φ12	+	+	-	+	-	+	+	+	6
Φ13	+	+	-	+	-	+	+	+	6
Φ14	+	+	-	+	-	+	+	+	6
Φ15	+	+	+	+	+	+	+	+	8
Φ16	+	+	-	+	-	+	+	-	5
Φ17	+	+	+	+	+	+	+	+	8
Φ18	+	+	-	+	-	+	+	+	6
Φ19	+	+	-	-	-	+	+	+	5
Φ20	+	+	+	+	+	+	+	+	8
Total	20	20	10	15	9	20	20	18	

P. carorovorum strain was susceptible to a phage is indicated with '+', was not susceptible to a phage indicated with '−'. The bottom row indicates the total amount of bacterial strains susceptible to a certain phage

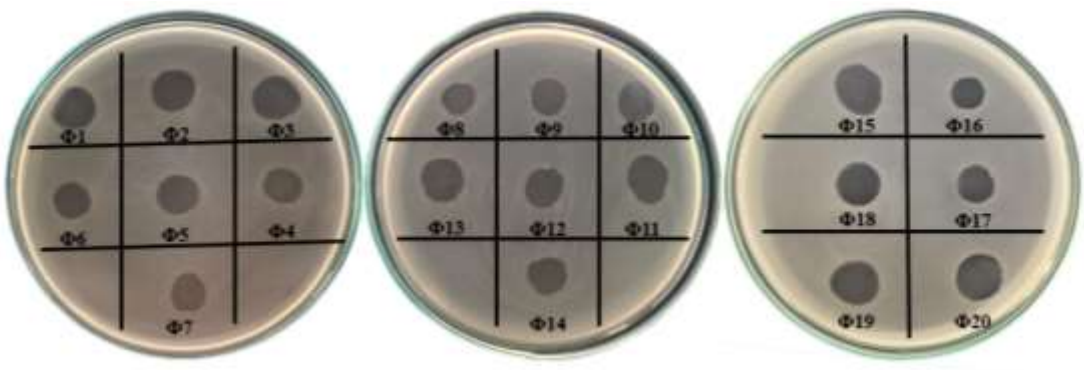

Figure 1. Susceptibility of twenty phages strain on *P. carotovorum* Pc2 at 24 hours

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From result of testing parasitic ability of phages on different *P. carotovorum* strain, therefore eight phages (Φ1, Φ2, Φ3, Φ5, Φ6, Φ15, Φ17, and Φ20) selected for further experiments.

3.3. Phage titer of different phage when cultured on *Pectobacterium carotovorum* Pc2

Log-transformed phage titers revealed highly significant differences ($p < 0,01$) among the 20 phages following 24 hours of incubation on *P. carotovorum*, with titers ranging from 7.36 to 8.73 log (PFU/mL). Phage Φ1 reached the highest titer (8.73 log PFU/mL), significantly surpassing all other phages. Phage Φ3 (8.47 log PFU/mL) produced a titer statistically equivalent to Φ5 (8.45 log PFU/mL), yet remained significantly higher than those of the remaining phage treatments.

Table 3. Log phage titer (PFU/mL) of eight different phages when cultured on *P. carotovorum Pc2*

Bacteriophages	Log phage titer (PFU/mL)
Φ1	8.73[a]
Φ2	7.70[d]
Φ3	8.47[b]
Φ5	8.45[b]
Φ6	8.18[c]
Φ15	7.88[d]
Φ17	7.36[e]
Φ20	8.30[bc]

Means followed by a different letter (a–e) in the same column differ significantly (Duncan's test; p-value < 0.01)

3.4. Plaque size of different bacteriophages when cultured on *Pectobacterium carotovorum* Pc2

Evaluation of plaque diameters on *P. carotovorum* Pc2 demonstrated that phages Φ3 and Φ5 exhibited superior degradation efficacy at all observation times. These two phages consistently produced the largest plaques, significantly exceeding those formed by other phages. Phages Φ1, Φ2, and Φ20 showed intermediate degradation capacity, with plaque diameters markedly larger than Φ6, Φ15, and Φ17 at 24, 36, and 48 hours.

Table 4. Plaque diameters (mm) of eight phages on *Pectobacterium carotovorum* Pc2

Phages	Plaque Diameter (mm)[1]		
	24 h	36 h	48 h
Φ1	3.83[a]	4.40[a]	4.57[b]
Φ2	3.77[a]	4.17[a]	4.50[b]
Φ3	4.33[a]	4.13[a]	4.90[ab]
Φ5	4.47[a]	4.93[a]	5.23[a]
Φ6	2.00[b]	2.00[b]	2.00[c]
Φ15	2.00[b]	2.00[b]	2.00[c]
Φ17	2.00[b]	2.00[b]	2.00[c]
Φ20	3.93[a]	4.33[a]	4.50[b]

Means followed by a different letter (a–c) in the same column do differ significantly (Duncan's test; p-value < 0.01)

According to Gallet et al. (2011), most bacteriophage plaques attain a characteristic size after incubation, and each plaque originates from a single viral particle. The typical circular plaque morphology simply reflects successive cycles of host-cell infection by progeny viruses radiating outward in all directions from the initial infection center. Consequently, different phages produce plaques of varying diameters, which mirror their distinct replication capacities over the same incubation period as well as their inherent host specificity.

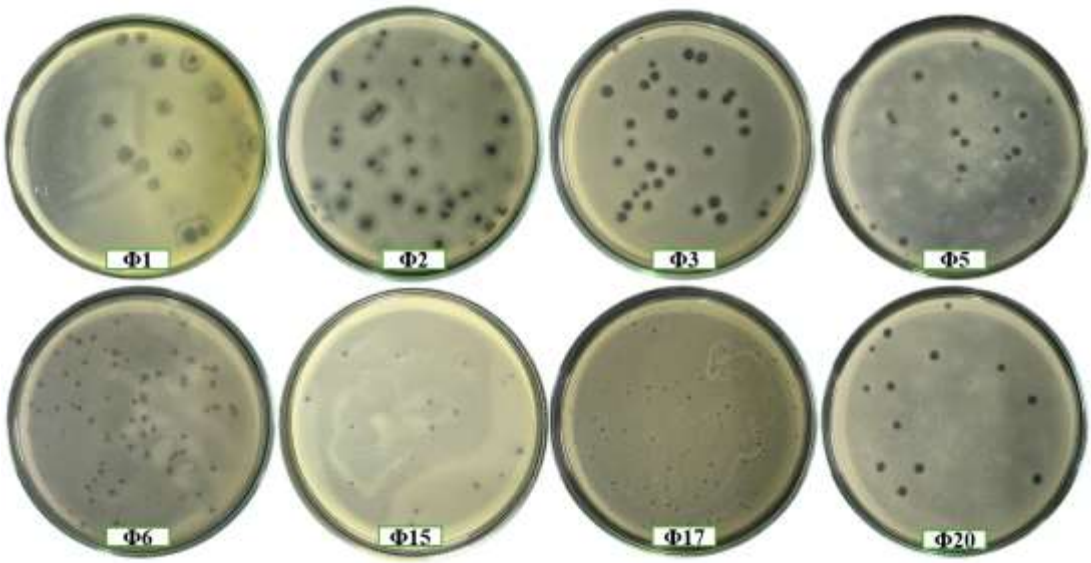

Figure 2. Morphology and plaque size of eight phages when cultured on *P. carotovorum* Pc2 at 48 hours

Based on phage titer and plaque diameter assays of eight phages tested on *P. carotovorum* Pc2, four phages Φ1, Φ3, Φ5, and Φ20 were selected for further biocontrol evaluation in greenhouse conditions against *P. carotovorum*, the causative agent of soft rot in cruciferous vegetables.

3.5. Greenhouse evaluation of bacteriophage biocontrol against soft rot on Bok Choy

At 2 days after inoculation (2 DAI), soft-rot symptoms such as water-soaked lesions and tissue maceration began to appear across treatments. Disease incidence ranged from 0.0% to 16.0%. The phage Φ20 treatment exhibited an incidence statistically equivalent to the chemical control (Starner 20WP) and significantly lower than both the untreated control and the phage Φ5 treatment. By 4 DAI, both Φ20 and Starner 20WP treatments demonstrated strong disease suppression, with incidences of 12.0% and 0.0%, respectively values significantly lower than those of the untreated control. At 6 DAI, the Φ3 and Φ20 treatments continued to maintain significantly reduced disease incidences compared to the untreated control; meanwhile, the Starner 20WP treatment remained completely disease-free.

In terms of the area under the disease progress curve (AUDPC), both phage Φ20 and Starner 20WP treatments exhibited significant disease suppression, reflected by

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lower AUDPC values compared with the control treatment.

Overall, only phage Φ20 effectively reduced disease incidence throughout the observation period, with phage Φ20 showing the highest and most consistent biocontrol efficacy.

Table 5. Disease incidence (%) and AUDPC of soft rot disease caused by *Pectobacterium carotovorum* on Bok choy following different treatments in greenhouse conditions

	Disease incidence (%)			AUDPC
	2 DAI	4 DAI	6 DAI	
Φ1	5.0^{ab}	20.0^{ab}	31.0^{ab}	76.0^{ab}
Φ3	9.0^{ab}	19.0^{ab}	23.0^{b}	70.0^{ab}
Φ5	16.0^{a}	21.0^{ab}	25.0^{ab}	83.0^{ab}
Φ20	3.0^{b}	12.0^{bc}	16.0^{b}	43.0^{bc}
Four-phage cocktail	8.0^{ab}	25.0^{ab}	31.0^{ab}	89.0^{ab}
Starner 20WP	0.0^{b}	0.0^{c}	0.0^{c}	0.0^{c}
Control	15.0^{a}	31.0^{a}	39.0^{a}	116.0^{a}
P-value	<0.01	<0.01	<0.01	<0.01

Means followed by the same letter (a-c) in the same column do not differ statistically among themselves by a Duncan's test ($p < 0.01$). DAI: days after inoculation with Pc2.

Figure 3. Level of soft rot disease on Bok choy caused by *Pectobacterium carotovorum* of different treatments at 6 days post-inoculation

4. CONCLUSION

All twenty bacteriophages were capable of parasitizing *Pectobacterium carotovorum* strains responsible for soft rot in cruciferous vegetables, with eight phages (i.e. Φ1, Φ2, Φ3, Φ5, Φ6, Φ15, Φ17, and Φ20) showing 100% parasitism across all eight bacterial strains. Four phages (Φ1, Φ3, Φ5, and Φ20) were selected based on their plaque diameters. In greenhouse biocontrol trials, phage Φ20 demonstrated the highest level of disease suppression nearly equivalent to the bactericide Starner 20 WP.

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