Malaysian Journal of Microbiology, Vol 20(1) 2024, pp. 15-23
DOI: http://dx.doi.org/10.21161/mjm.230261

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Isolation and toxicity of *Xanthomonas oryzae* pv. *oryzae* in rice and a collection of phages against the pathogen

Ngoc Quynh Anh Pham[1,2], Vo Thanh Phuc[1,2], Pham Do Tra My[1,2], Le Thi My Duyen[1,2], To Hue Ngoc[1,2], Nguyen Minh Thien[1,2], Nguyen Hai Duong[1,2], Le Thi Thuy Tien[1,2], Le Phi Nga[1,2] and Hoang Anh Hoang[1,2]*

[1]Faculty of Chemical Engineering, Ho Chi Minh City University of Technology (HCMUT), 268 Ly Thuong Kiet, District 10, Ho Chi Minh City, Vietnam.
[2]Vietnam National University Ho Chi Minh City, Linh Trung Ward, Thu Duc District, Ho Chi Minh City, Vietnam.
Email: hoang.a.hoang@hcmut.edu.vn

Received 14 April 2023; Received in revised form 7 September 2023; Accepted 12 September 2023

ABSTRACT

Aims: The objective of the study was to isolate bacteriophages and conduct a comprehensive analysis of their potential against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strains in the Mekong Delta, Vietnam.
Methodology and results: Twelve *Xoo* strains were isolated from rice fields located in the Mekong Delta, Vietnam. Among these strains, three strains *Xoo* L019, L020 and L024, showed the highest disease index of bacterial blight. Four phages specific to *Xoo* were isolated from soil, water and leaf samples, and their morphologies were determined. In a test against 12 *Xoo* strains, phage L541, MLA23 or W41 could infect 10 of the 12 *Xoo* strains, while phage LBH01 could infect 8 of the 12 *Xoo* strains. The stability of the phages to pH, organic solvents, UV-A and UV-B was also evaluated.
Conclusion, significance and impact of study: The initial characterization of the phages indicates their potential as biocontrol agents against bacterial blight in rice. The study is one of the very first studies about *Xoo* phages in rice in Vietnam.

Keywords: Bacterial blight, bacteriophage, biocontrol, stability, toxicity

INTRODUCTION

Rice is an essential crop that sustains about 3.5 billion people worldwide, mainly in Asia (Gnanamanickam, 2009). In Vietnam, the government always maintained rice production at 41-43 million tons per year to ensure food security. However, rice production is constantly exposed to the risk of seasonal disease, the leading cause of yield reduction. Common diseases of rice are blast, sheath blight, sheath rot, bacterial blight and rice tungro disease. Among these, bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae (Xoo)* is a severe disease that can reduce rice yield by 50-60% (Gnanamanickam, 2009). The severity of losses incurred due to the disease necessitates developing efficient, environmentally friendly and cost-effective strategies. On the one hand, the most common solution is pesticides, but this pollutes the water, soil and air environments (Kim *et al.*, 2017). On the other hand, the most researched solution is using *Xoo* amensalism bacteria such as *Bacillus* spp., *Actinomyces* and *Pseudomonas aeruginosa* (Hop *et al.*, 2014; Cheng *et al.*, 2016; Yasmin *et al.*, 2017). However, because the microorganisms used in biological control only inhibit the reproduction of pathogenic microorganisms and cannot eliminate *Xoo* directly, their efficiency is lower than expected (Adachi *et al.*, 2012).

Bacteriophage has been shown as a potential solution against BB due to its high specificity to pathogenic bacteria and ecologically friendly. Since the first study using bacteriophages to control *Xoo* in 1969 (Kuo *et al.*, 1969), many follow-up studies have been carried out and focused on three main goals in general (Nakayinga *et al.*, 2021; Liu *et al.*, 2022). The first is the isolation of bacteriophages. The second is the characterization of isolated bacteriophages. The characteristics are often investigated, such as burst size, morphology, the stability of the phage in different pH, temperature and UV conditions, etc. (Adhikari and Basnyat, 1999; Ogunyemi *et al.*, 2019). The third is to evaluate the ability of bacteriophages to control BB on a laboratory scale (Giang *et al.*, 2014; Jain *et al.*, 2023; Jiang *et al.*, 2023).

Moreover, the effectiveness of disease prevention and treatment depends on (1) the virulence and resistance of pathogenic bacteria in different geographical areas of cultivation with different climates, weather and soil; (2) the infectivity and control activities of bacteriophages isolated from different cultivation areas were also different; (3)

***Corresponding author**

ISSN (print): 1823-8262, ISSN (online): 2231-7538

different disease prevention and treatment procedures. The current study focused on the isolation and toxicity analysis of *Xoo* strains in the Mekong Delta, Vietnam. From this bacterial collection, we proceeded to isolate some potential phages and analyze the morphology, host range and stability of these phages. The study is one of the very first studies about *Xoo* phages in rice in Vietnam, and it will be a vital premise for selecting suitable phages to achieve the goal of using phage biocontrol against BB in Vietnam.

MATERIALS AND METHODS

Isolation of *Xoo*

Rice leaves showing typical symptoms of bacterial blight were collected from rice-growing areas in Ho Chi Minh City, Long An and Dong Nai. Leaf samples were separated and placed in ziplock bags. Sample bags labeled with the sample name, date and location were transported to the laboratory and stored at 4 °C until used for bacterial isolation. Leaf samples were washed with clean water, blotted dry and cut into small pieces of about 5-7 cm. Sodium hypo chloride (1%) was used for 3 min as a disinfectant for the leaf surface. The leaf samples were then rinsed with sterile distilled water 2-3 times before being blotted dry with sterile paper. The leaf was then cut into smaller pieces measuring 5 × 5 mm and placed in 1.5 mL tubes containing 1 mL of sterile distilled water for 15 min to release bacteria from the sample. Next, 100 µL of these were cultured onto Petri dishes containing Modified Wakimoto Agar and incubated at 30 °C for 72 h.

Bacterial isolates were subjected to the colony PCR method with *Xoo*-specific primer pairs, XOO290F (5'-GCGCACCGAGTATTCCTA-3')/ XOO290R (5'-CTTCGCCGGTCCAGATGA-3') and XORF (5'-GCATGACGTCAT CGTCCTGT-3')/ XORR2 (5'-CTCGGAGCTATA TGCCGTGC-3') (Lien *et al.*, 2012). PCR program was conducted, including initiation at 95 °C for 3 min, followed by 35 cycles at 95 °C for 15 sec, 56 °C for 30 sec, 72 °C for 30 sec and the final extension at 72 °C for 5 min. The amplified products were separated into a 1% agarose gel for 30 min at 80 V with the gelred 6× DNA stain. Furthermore, bacterial DNAs were also extracted according to the procedure described by Sambrook *et al*. (1989) to be used for bacterial identification by entire 16S rRNA sequencing with primers 8F (5'-AGAGTTTGATCATGGCTCAG-3')/ 15R (5'-AAGGAGGTGATCCAACCGCA-3') (Sambrook *et al.*, 1989). The 16S rRNA sequences of selected bacteria were compared with sequences in the nucleotide database of the NCBI library through the BLAST tool.

Toxicity test of *Xoo* in rice

The study was conducted at the Southern Research Center for Plant Protection in Long An province, Vietnam. The Jasmin 85 rice variety provided by the Cuu Long Delta Rice Research Institute was used to characterize the toxicity of the *Xoo* strains. The positive control was the *Xoo* strain provided by the Vietnam Type Culture Collection (VTCC 12268). The seeds were pre-germinated at room temperature for 48 h. The plants were grown in 17.5 × 12 cm plastic pots containing 1 kg of soil. Seeds were sown in pots, 20 seeds in each pot, 10 seeds in a row. After 7 days of sowing, prune 5 plants per row. The amount of fertilizer was calculated according to the area of plastic pots with the formula 120 N, 40 P_2O_5, and 30 K_2O kg/ha (Giang *et al*., 2014). The water level in the pot was about 2 cm. The plants were protected from pest attacks. The inoculation was done after 45 days of sowing rice seeds. *Xoo* strains were grown on Petri dishes containing Wakimoto medium for 4 days, then 10 mL of sterile physiological saline (0.9%) was added to collect bacterial suspensions. The inoculum density was adjusted to 10^7, 10^8 and 10^9 CFU/mL.

The plants were artificially inoculated by clipping the tips of the leaves (about 2 cm from the tip) with sterilized scissors dipped in a bacterial suspension (Kauffman *et al.*, 1973). The treated leaves were marked to record the results. Each bacterial strain was inoculated on 5 rice plants, and 2 leaf blades per plant were inoculated. Control plants were inoculated similarly with sterile distilled water. The change of rice leaves and disease reactions were evaluated at 7 and 14 days after inoculation, and disease severity was assessed based on the disease rate and disease index.

The disease rate (%)
= (Number of diseased leaves/Total number of leaves in the treatment) × 100

The disease index (%)
$= (9n_9 + 7n_7 + 5n_5 + 3n_3 + 1n_1)/9N \times 100$

n_1, n_3, n_5, n_7: Corresponding to the number of leaves whose diseased area was less than 1%, 5%, 25%, 50%; n_9: The number of leaves whose diseased area was greater than 50%; N: Total number of leaves in the treatment.

Statistical analysis

Each treatment was conducted with five replications and arranged in a completely randomized design. The data was analyzed using one-way analysis of variance (ANOVA) by MSTAT-C software and the significant differences between means were determined by the LSD-test at $p \leq 0.05$.

Phage isolation

Soil, water and leaf samples from infected fields in the mentioned province were also used to isolate bacteriophages. Even though sample preservation conditions remained the same at 4 °C until used, the pre-treatment methods for each sample type were different. Five (5) g of soil sample were placed in a falcon tube. Five mL of distilled water and chloroform at 5% (w/v) were added into the tube. The mixture was then vortexed for 5

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

min and centrifuged at 2,432× *g* for 10 min at room temperature to obtain the supernatant and discard the soil and chloroform layers (My *et al*., 2023). Next, the supernatant was centrifuged at 9,727× *g* for 5 min at 4 °C to discard the remaining chloroform. The resulting supernatant was passed through a 0.22 µm filter and the filtrate was used in phage enrichment and plaque assay. For the water sample, 10 mL were added to the falcon tube, and chloroform was added at a rate of 2% v/v and mixed by the vortex. The sample was then subjected to two steps of centrifugation and filtration, like the treatment of soil samples. Leaf samples were washed, dried and crushed in a porcelain mortar. Then, the sample was placed in a 50 mL falcon tube with 5 mL of sterile distilled water. The samples were centrifuged twice and filtered once, with the same conditions as the two other types of samples.

The filtrate was added to a falcon tube containing 100 µL of log-phase *Xoo* bacterial culture and 9 mL of TSB medium. The mixture was shaken at 150 rpm for 24 h at 30 °C and was then centrifuged at 9,727× *g* for 5 min at 4 °C. The resulting supernatant was passed through a 0.22 µm pore size filter and the filtrate was subjected to a plaque assay. Next, a mixture of 100 µL of the filtrate and 200 µL of log-phase bacterial culture was added to 3 mL of molten 0.5% TSA (maintained at 42 °C) and poured over a 1.5% Luria-Bertani (LB) agar plate. After incubation overnight at 30 °C, a single transparent plaque was selected from the plate, suspended in SM buffer [100 mM NaCl, 10 mM $MgSO_4$, 0.01% gelatine and 50 mM Tris-HCl (pH 7.5)], incubated overnight at 4 °C and passed through a 0.22 µm filter (Hoang *et al*., 2019). The resulting filtrate was subjected to the above protocol three times to purify the phage.

The morphologies of bacteriophages were analyzed by Transmission Electron Microscopy (TEM). First, a phage sample with a high titer (10^{10} PFU/mL) was generated and then negatively stained with 5% uranyl acetate. Each sample was examined using a transmission electron microscope (JEOL JEM-1010, Japan) operating at 80 kV of voltage and an instrumental magnification of 25,000-30,000 at the Vietnam National Institute of Hygiene and Epidemiology. Based on morphology and size, phages would be classified into appropriate families (Ackermann, 2009).

Phage stability test

Stability of phages to pH, organic solvents, UVA and UVB was carried out. To determine the phage stability at different pH values, the pH of TSB was adjusted using either 1 M HCl or 1 M NaOH to attain solutions with a pH of 3, 4, 5, 6, 7, 8, 9, 10 and 11 (Xuan *et al*., 2018). The phage suspension, at approximately 10^8 PFU/mL, was mixed with an equal volume of the TSB and incubated at 30 °C for 24 h. To evaluate the phage stability in SM buffer as a control and in organic solvents, a volume of the phage at approximately 10^8 PFU/mL was mixed with an equal volume of SM buffer or the organic solvent, i.e., ethanol, chloroform, or diethyl ether and incubated at 30 °C for 1 h (Xuan *et al*., 2018). After incubation, the double agar-layer method calculated phage titers by serial dilution. The UV exposure of each phage was conducted at 306 nm (UV-B, model Philips PL-S 9W/01/2P) and 365 nm (UV-A, model Phillips Actinic BL TL-D 15W). The phage stock was diluted in SM buffer to a final concentration of approximately 8 $\log_{10}$ (PFU/mL). Two mL of phage suspension were placed in an Φ-6 mm Petri dish. The distance from the light source to the Petri dish was approximately 30 cm. Sampling was conducted every 10 min. The sample was subjected to a plaque assay to determine phage titer. The experiment was conducted in triplicate.

Host range determination of phages

The host range of phages was determined against 12 *Xoo* strains. The susceptibility of which was examined using a drop plaque assay. On a 1.5% LB agar plate, a 100 µL aliquot of an overnight bacterial culture was combined with 3.0 mL molten 0.5% TSB agar (kept at 42 °C). Each plate got 2 µL of phage stock after two min (approximately 10^9 PFU/mL). The plates were incubated overnight at 30 °C before being checked for clear plaque on the bacterial lawn (My *et al*., 2023).

RESULTS AND DISCUSSION

A collection of *Xoo*

Single colonies of light yellow, circular, convex, smooth and mucilaginous color appeared on Petri dishes containing Modified Wakimoto Agar (Lien *et al.*, 2012). A total of 30 isolates expected to be *Xoo* were selected and purified. The PCR with specific primer pair XOO290 F/R resulted in electrophoresis bands with the expected size of 290 bp in the case of 12/30 isolates: L005, L006, L008, L017, L019, L020, L021, L022, L023, L024, L025, L027 (Table 1). The PCR with the specific primer pair XORF/XORR2 resulted in electrophoresis bands with the expected size of 470 bp in these 12 isolates. The 16S rRNA sequencing results indicated that all 12 isolates were *Xanthomonas oryzae*.

Toxicity of *Xoo* in rice

Some *Xoo* strains and VTCC 12268 showed typical symptoms of disease in the experimental rice plants after being inoculated. After 7 days of inoculation, the lesions were grayish-green. As the inoculum density increased, disease symptoms became more severe. With an increase in inoculum density, the disease symptoms escalated in severity. Affected rice leaves displayed water-soaked lesions, manifesting as narrow, dark green streaks that tended to merge, forming larger, irregularly shaped lesions extending along the leaf veins. Over time, the color of the lesions transitioned from dark green to brown or reddish-brown, signifying disease progression. After 14 days of inoculation, the leaves turned from yellow

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 20(1) 2024, pp. 15-23
DOI: http://dx.doi.org/10.21161/mjm.230261

Table 1: Disease rate of *Xoo* strains at different inoculations.

Strains	Disease rate (%)					
	After 7 days of inoculation			After 14 days of inoculation		
	10^7	10^8	10^9	10^7	10^8	10^9
L005	16.67 ^{hi}	33.33 ^{e}	80.95 ^{bc}	90.48 ^{ab}	92.86 ^{abc}	92.86 ^{a}
L006	19.05 ^{hi}	35.71 ^{e}	64.29 ^{e}	95.24 ^{a}	95.24 ^{ab}	97.62 ^{a}
L008	26.19 ^{gh}	35.71 ^{e}	88.09 ^{abc}	73.81 ^{cde}	73.81 ^{d}	88.09 ^{a}
L017	80.95 ^{ab}	85.71 ^{ab}	90.47 ^{ab}	80.95 ^{bcd}	85.71 ^{abcd}	90.47 ^{a}
L019	76.19 ^{ab}	97.62 ^{a}	97.62 ^{a}	76.19 ^{cde}	97.62 ^{a}	97.62 ^{a}
L020	85.71 ^{a}	90.47 ^{ab}	95.24 ^{a}	85.71 ^{abc}	90.47 ^{abc}	95.24 ^{a}
L021	52.38 ^{ef}	61.91 ^{d}	78.57 ^{cd}	73.81 ^{cde}	90.48 ^{abc}	97.62 ^{a}
L022	61.91 ^{de}	83.33 ^{bc}	88.09 ^{abc}	76.19 ^{cde}	95.24 ^{ab}	95.24 ^{a}
L023	64.29 ^{cd}	71.43 ^{cd}	83.33 ^{bc}	69.05 ^{de}	80.95 ^{cd}	92.86 ^{a}
L024	78.57 ^{ab}	95.24 ^{ab}	95.24 ^{a}	85.71 ^{abc}	97.62 ^{a}	97.62 ^{a}
L025	14.29 ^{i}	16.67 ^{f}	45.24 ^{f}	38.09 ^{f}	47.62 ^{e}	73.81 ^{b}
L027	50.00 ^{f}	64.29 ^{d}	83.33 ^{bc}	73.81 ^{cde}	85.71 ^{abcd}	92.86 ^{a}
VTCC 12268 (+)	30.95 ^{g}	64.29 ^{d}	78.57 ^{cd}	66.67 ^{e}	83.33 ^{bcd}	88.09 ^{a}
Negative control	0	0	0	0	0	0
CV (%)	13.8	11.53	8.35	10.19	9.05	8.51
$LSD_{0.05}$	11.85	12.59	11.58	12.42	12.74	13.01

Different letters within a column indicate significant differences with *p*-value ≤ 0.05.

Table 2: Disease index of *Xoo* strains at different inoculations.

Strains	Disease Index (%)					
	After 7 days of inoculation			After 14 days of inoculation		
	10^7	10^8	10^9	10^7	10^8	10^9
L005	1.85 ^{hi}	3.7 ^{e}	8.99 ^{bc}	10.05 ^{g}	15.61 ^{h}	25.13 ^{f}
L006	2.12 ^{hi}	3.97 ^{e}	7.14 ^{e}	10.58 ^{g}	28.57 ^{g}	29.37 ^{ef}
L008	2.91 ^{gh}	3.97 ^{e}	9.79 ^{abc}	8.73 ^{g}	9.79 ^{hi}	23.55 ^{f}
L017	8.99 ^{ab}	9.52 ^{ab}	10.05 ^{ab}	79.37 ^{ab}	85.18 ^{bc}	89.95 ^{ab}
L019	8.47 ^{ab}	10.85 ^{a}	10.85 ^{a}	67.73 ^{cd}	94.44 ^{a}	96.03 ^{a}
L020	9.52 ^{a}	10.05 ^{ab}	10.58 ^{a}	82.01 ^{a}	85.71 ^{bc}	92.6 ^{ab}
L021	5.82 ^{ef}	6.88 ^{d}	8.73 ^{cd}	35.19 ^{f}	45.5 ^{f}	62.7 ^{d}
L022	6.88 ^{de}	9.26 ^{bc}	9.79 ^{abc}	58.73 ^{d}	74.07 ^{d}	73.02 ^{c}
L023	7.14 ^{cd}	7.94 ^{cd}	9.26 ^{bc}	45.77 ^{e}	60.32 ^{e}	85.98 ^{b}
L024	8.73 ^{ab}	10.58 ^{ab}	10.58 ^{a}	67.73 ^{cd}	91.27 ^{ab}	94.98 ^{a}
L025	1.59 ^{i}	1.85 ^{f}	5.03 ^{f}	4.23 ^{g}	7.94 ^{hi}	24.6 ^{f}
L027	5.55 ^{f}	7.14 ^{d}	9.26 ^{bc}	10.32 ^{g}	16.4 ^{h}	32.01 ^{ef}
VTCC 12268	3.44 ^{g}	7.14 ^{d}	8.73 ^{cd}	9.52 ^{g}	28.31 ^{g}	35.18 ^{e}
Negative control	0	0	0	0	0	0
CV (%)	13.78	11.53	8.35	13.81	9.88	8.91
$LSD_{0.05}$	1.315	1.399	1.287	9.211	8.47	8.907

Different letters within a column indicate significant differences with *p*-value ≤ 0.05.

to straw-colored and wilted (Figure 1). No disease symptoms were observed on leaves inoculated with sterile distilled water (negative control).

The results indicated that the highest disease rate and disease index of *Xoo* strains were obtained when inoculated with the highest bacterial concentration (10^9 CFU/mL). The results in Table 1 show that, at a concentration of 10^9 CFU/mL, strain VTCC 12268 and *Xoo* strains had high disease rates, except for strain L025, which had a disease rate of only 73.81%). However, the disease indexes of *Xoo* strains were different, as shown in Table 2. Strains L017, L019, L020 and L024 had high disease indexes of 89.95%, 96.03%, 92.6% and 94.98%, respectively. In contrast, the disease index of strain VTCC 12268 (the positive control) was significantly lower, at only 35.18%.

According to previous reports, *Xoo* strains are diverse and have different toxicity (Noda *et al*., 1999; Pandey *et al*., 2017; Kogeethavani *et al*., 2021). Kogeethavani *et al.* (2021) found that 21 strains of *Xoo* isolated from Malaysia had a high virulence diversity in the Near Isogenic rice Lines. Race R20, which was identified as dominant, was virulent to most of the Near Isogenic rice Lines. In this study, *Xoo* strains were isolated from different regions in southern Vietnam. The collection site had an impact on the diversity of pathotypes (Furuya *et al*., 2012).

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 20(1) 2024, pp. 15-23
DOI: http://dx.doi.org/10.21161/mjm.230261

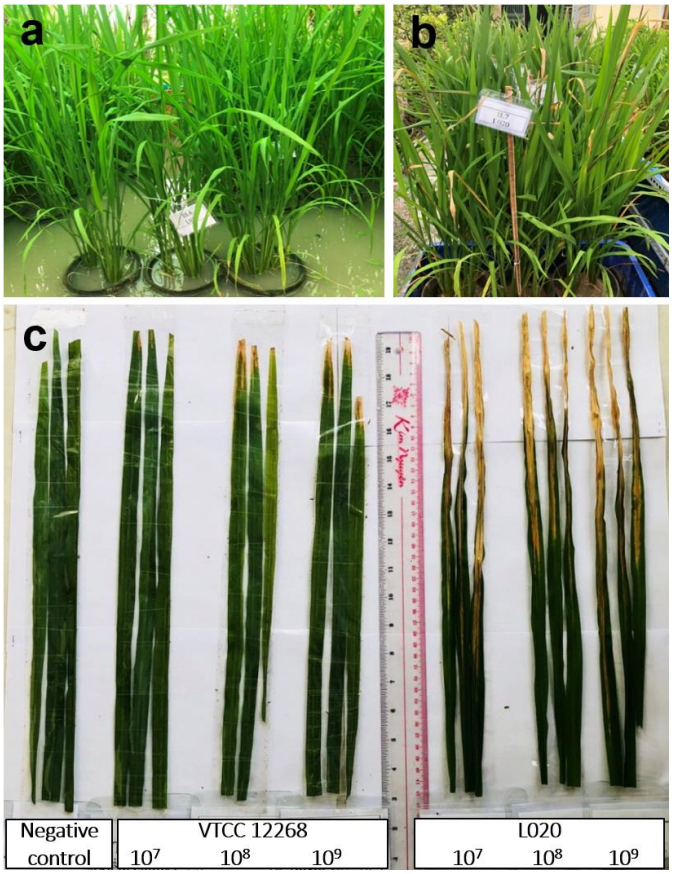


Figure 1: Bacterial leaf blight disease symptoms on rice plants. a) Rice plants before inoculation (45 days after sowing); b) Rice plants after 14 days of inoculation; c) Lesions on leaves after 14 days of inoculation.

Isolation and morphology of phages

Four bacteriophages, L541, W41, MLA23 and LBH01 were isolated and purified from the samples (Table 3). Phage L541 was isolated from a leaf sample. It showed a siphovirus morphology with a long tail (~135 nm) and an icosahedral head (~58 nm). Phage W41 had an icosahedral head (~58 nm) and a long tail (~81 nm). The diameter of the icosahedral head and tail length measured on the MLA23 particle were approximately 61 nm and 112 nm, respectively. Both phages had the same morphology as myovirus phages. The fourth phage LBH01, had an Autographiviridae morphology with an icosahedral head with a diameter of approximately 53 nm.

Stability of the phages

The pH stability analysis showed that all four phages were stable over a wide pH range of 4 to 11 after 24-h incubation at 30 °C (Figure 2A). Although the titer of phages remained unchanged in most pH variations, sharp drops in titer were observed at pH 3. While there was no viable phage at pH 3 in experiments with L541 and LBH01, the concentration of phage MLA23 decreased by only 1 log10 unit compared to that at other pH values. Therefore, these four phages were considered more stable at low pH values than published *Xanthomonas* phages (Ranjani *et al.*, 2018; Nakayinga *et al.*, 2021).

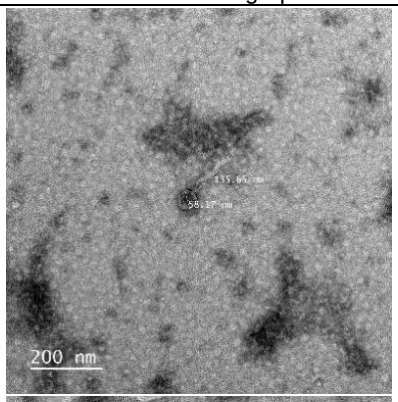
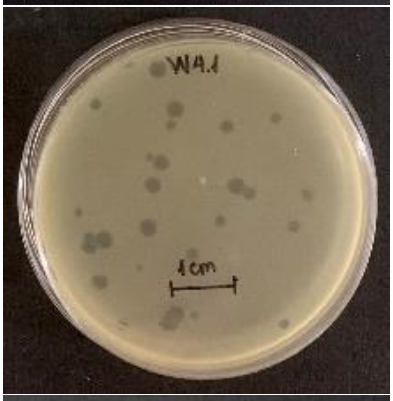
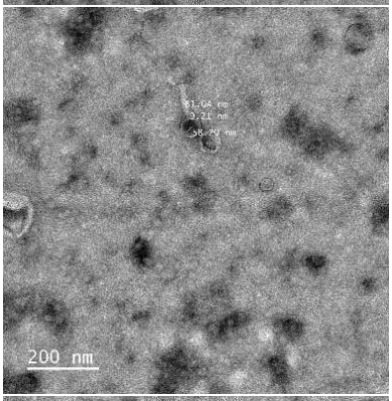
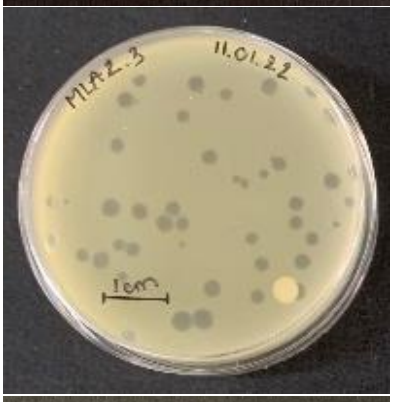
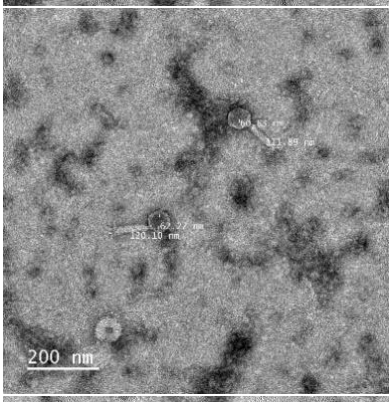
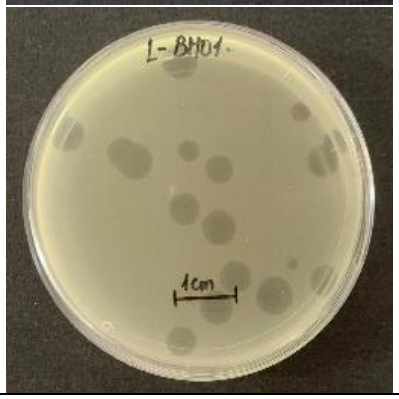
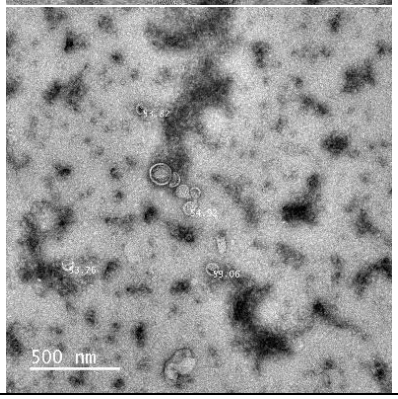
The result of phage stability analysis in organic solvents demonstrated that all four phages were highly resistant to chloroform and diethyl ether for 1-h incubation (Figure 2B). There was only a slight decline at a significant level in the phage L541 titer after exposure to chloroform. However, no phage survived in ethanol except phage W41, which remained at approximately 10 PFU/mL.

According to Figures 2C and 2D, it is clarified that phage viability was less affected by UV-A and UV-B light in a 1-h exposure. Viable phage counts of L541 started to decline by ~0.1 log 10 units after 30 min in both UV conditions. The same result was observed in phage W41 under UV-B light. On the contrary, the phage titers of MLA23 and LBH01 remained at their initial values during the experiment. Phage vB_XooS_NR08 isolated by Jain *et al*. also showed high viability with direct exposure to sunlight for 120 min, but the titer dramatically reduced after 150 min (Jain *et al.*, 2023). Natural sunlight's UV levels (UV-A and UV-B) changed throughout the day and reached their maximum value in the early afternoon. Preventing daylight exposure or applying protective

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 20(1) 2024, pp. 15-23
DOI: http://dx.doi.org/10.21161/mjm.230261

Table 3: Top agar overlay showing plaque morphology of phages and their respective electron micrographs.

Phage	Plaque morphology	Electron micrograph
L541		
W41		
MLA23		
LBH01		

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 20(1) 2024, pp. 15-23
DOI: http://dx.doi.org/10.21161/mjm.230261

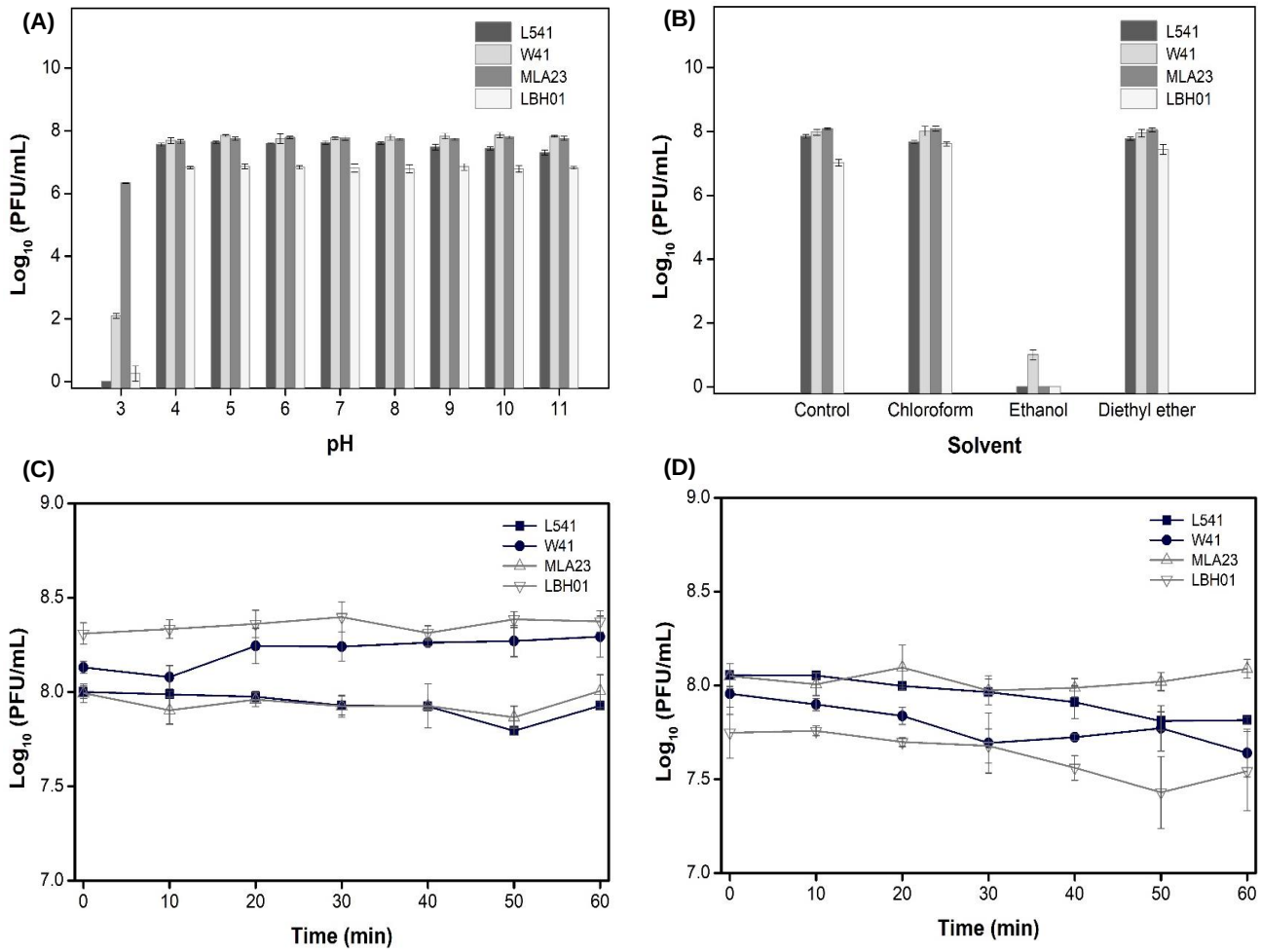


Figure 2: Viability of the phages at various pHs (A), in the presence of various organic solvents (B), under UVA (C) and UVB (D) exposure. Error bars indicate 95% confidence intervals for the averaged values (n=3).

Table 4: Host range of the phages against the bacterial isolates.

Bacterial strains[a]	Plaque formation of phages			
	L541	MLA23	LBH01	W41
Xoo L005	+	+	+	+
Xoo L006	+	+	+	+
Xoo L008	-	+	-	+
Xoo L017	+	+	+	+
Xoo L019	+	-	-	-
Xoo L020	+	+	+	+
Xoo L021	+	+	+	+
Xoo L022	+	+	+	+
Xoo L023	+	+	+	+
Xoo L024	+	+	+	+
Xoo L025	+	-	-	-
Xoo L027	-	+	-	+

(+): Susceptible; (-): Not susceptible; MTCCa: Microbial Type Culture Collection.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

components are strategies to improve the effectiveness of phage-based biocontrol (Iriarte *et al.*, 2007; Jain *et al.*, 2023).

Host range of phages

The host range of four phages was determined using 12 bacterial isolates from *Xoo* collection (Table 4). Phage L541 formed clear zones in cultures of 10/12 *Xoo* strains, implying that it could infect these *Xoo* strains. It did not infect *Xoo* L008 or *Xoo* L027. Phage MLA23 or W41 could infect 10/12 *Xoo* strains except *Xoo* L019 and *Xoo* L025. Phage LBH01 created clear zones in cultures of 8/12 *Xoo* strains and could not infect four strains: L008, L019, L025 and L027. There was no single phage that could infect all 12 *Xoo* strains. Bacteriophages have specific surface proteins that recognize and bind to receptors on host cells. If the host cells lack the appropriate receptors, the phage cannot initiate the infection. Genetic variations among *Xoo* strains may lead to differences in the distribution of these receptors, rendering some strains resistant to specific phages (Silva *et al.*, 2016). To enhance the efficiency of phage biocontrol against bacterial hosts, a phage cocktail is generally considered (Tanji *et al*., 2004; Hoang and Pham, 2021). A phage cocktail is the use of different phages that exploit different host bacterial receptors and thus broaden the phage host range. For instance, the combination of L541 and MLA23 is expected to infect 12/12 *Xoo* strains in Table 4 and increase the efficiency of the phage biocontrol. In addition, the regular emergence of phage-resistant bacteria is a significant obstacle to phage therapy. Using a phage cocktail to inactivate phage-resistant bacteria efficiently addresses the problem (Tanji *et al*., 2004; Filippov *et al*., 2011).

CONCLUSION

In this study, a collection of *Xoo* strains was isolated from Vietnam rice fields, and their toxicity was evaluated based on the disease index of the bacterial blight on rice. Four phages specific to native *Xoo* were successfully isolated. Their stability under different conditions such as pH, organic solvents, UV-A and UV-B and their ability to inhibit various native *Xoo* strains were analyzed. The results indicated the potential of the phages as effective biocontrol agents against bacterial blight in rice in Vietnam.

ACKNOWLEDGEMENTS

This research was funded by the Department of Science and Technology of HCMC, Vietnam, under contract number 110/2020/HĐ-QPTKHCN.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

REFERENCES

Ackermann, H. W. (2009). Phage classification and characterization. *In*: Bacteriophages: Methods and Protocols, Volume 1: Isolation, Characterization, and Interactions. Clokie, M. R. J. and Kropinski, A. M. (eds.). Humana Press, Totowa, NJ. **pp. 127-140.**

Adachi, N., Tsukamoto, S., Inoue, Y. and Azegami, K. (2012). Control of bacterial seedling rot and seedling blight of rice by bacteriophage. *Plant Disease* **96(7), 1033-1036.**

Adhikari, T. B. and Basnyat, R. (1999). Phenotypic characteristics of *Xanthomonas campestris* pv. *campestris* from Nepal. *European Journal of Plant Pathology* **105(3), 303-305.**

Cheng, J., Jaiswal, K. S., Yang, S. H. and Suh, J. W. (2016). Endophytic *Bacillus subtilis* MJMP2 from Kimchi inhibits *Xanthomonas oryzae* pv. *oryzae*, the pathogen of rice bacterial blight disease. *Journal of Applied Biological Chemistry* **59(2), 149-154.**

Filippov, A. A., Sergueev, K. V., He, Y., Huang, X. Z., Gnade, B. T., Mueller, A. J. *et al.* (2011). Bacteriophage-resistant mutants in Yersinia pestis: identification of phage receptors and attenuation for mice. *PLoS ONE* **6(9), e25486.**

Furuya, N., Taura, S., Goto, T., Thuy, B. T., Ton, P. H., Tsuchiya, K. and Yoshimura, A. (2012). Diversity in virulence of *Xanthomonas oryzae* pv. *oryzae* from Northern Vietnam. *Japan Agricultural Research Quarterly: JARQ* **46(4), 329-338.**

Giang, T. N. T., Nga, N. T. T. and Kieu, T. D. T. (2014). Isolation bacteriophages and evaluation of their effect in controlling bacterial leaf blight disease on rice caused by *Xanthomonas oryzae* pv. *oryzae*. *Can Tho University Journal of Science* **194-203.**

Gnanamanickam, S. S. (2009). Rice and its importance to human life. *In*: Biological Control of Rice Diseases. Gnanamanickam, S. S. (eds.). Springer, Dordrecht. **pp. 1-11.**

Hoang, H. A., Xuan, T. T. T., Nga, L. P. and Oanh, D. T. H. (2019). Selection of phages to control *Aeromonas hydrophila* – An infectious agent in striped catfish. *Biocontrol Science* **24(1), 23-28.**

Hoang, H. A. and Pham, D. T. M. (2021). Phage cocktails to inactivate *Edwardsiella ictaluri*, an infectious agent in striped catfish *Pangasianodon hypophthalmus*. *Chemical Engineering Transactions* **89, 535-540.**

Hop, D. V., Phuong Hoa, P. T., Quang, N. D., Ton, P. H., Ha, T. H., Hung, N. V. *et al.* (2014). Biological control of *Xanthomonas oryzae* pv. *oryzae* causing rice bacterial blight disease by *Streptomyces toxytricini* VN08-A-12, isolated from soil and leaf-litter samples in Vietnam. *Biocontrol Science* **19(3), 103-111.**

Iriarte, F. B., Balogh, B., Momol, M. T., Smith, L. M., Wilson, M. and Jones, J. B. (2007). Factors affecting survival of bacteriophage on tomato leaf surfaces. *Applied and Environmental Microbiology* **73(6), 1704-1711.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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. *Frontiers in Microbiology* **14, 1084025.**

Jiang, H., Li, C., Huang, X., Ahmed, T., Ogunyemi, S. O., Yu, S. *et al.* (2023). Phage combination alleviates bacterial leaf blight of rice (*Oryza sativa* L.). *Frontiers in Plant Science* **14, 1147351.**

Kauffman, H. E., Reddy, A. P. K., Hsieh, S. P. Y. and Merca, S. D. (1973). An improved technique for evaluating resistance of rice varieties to *Xanthomonas oryzae*. *Plant Disease Reporter* **57(6), 537-541.**

Kim, K. H., Kabir, E. and Jahan, S. A. (2017). Exposure to pesticides and the associated human health effects. *Science of The Total Environment* **575, 525-535.**

Kogeethavani, R., Fatin, N. A., Suzianti, I. V. and Erwan, S. S. (2021). Characterization of pathogenic variability of *Xanthomonas oryzae* pv. *oryzae* isolates causing bacterial leaf blight disease in Malaysian rice granaries. *Australasian Plant Pathology* **50(3), 293-298.**

Kuo, T. T., Huang, T. C. and Chow, T. Y. (1969). A filamentous bacteriophage from *Xanthomonas oryzae*. *Virology* **39(3), 548-555.**

Lien, N. T., Pha, N. T. and Mai, T. T. X. (2012). Isolation and identification of bacteria caught bacterial leaf blight (*Xanthomonas oryzae* pv. *oryzae*) by multiplex PCR technique. *Can Tho University Journal of Science* **(23a), 155-164.**

Liu, J., Wang, H., Chia, S. L. and Tan, G. H. (2022). Screening and formulation of novel carriers for *Xanthomonas* bacteriophage to control bacterial leaf blight disease. *Malaysian Journal of Microbiology* **18(5), 490-504.**

My, P. D., Vinh, T. Q., Ngoc, T. H., Anh, P. N., Duyen, L., Thien, N. M. *et al.* (2023). Complete genome sequence of a novel lytic phage of *Xanthomonas oryzae* pv. *oryzae*, the bacterial leaf blight pathogen in rice. *Archives of Virology* **168(6), 157.**

Nakayinga, R., Makumi, A., Tumuhaise, V. and Tinzaara, W. (2021). *Xanthomonas* bacteriophages: A review of their biology and biocontrol applications in agriculture. *BMC Microbiology* **21(1), 291.**

Noda, T., Van Du, P., Van E, L., Dinh, H. D. and Kaku, H. (1999). Pathogenicity of *Xanthomonas oryzae* pv. *oryzae* strains in Vietnam. *Japanese Journal of Phytopathology* **65(3), 293-296.**

Ogunyemi, S. O., Chen, J., Zhang, M., Wang, L., Masum, M. M. I., Yan, C. *et al.* (2019). Identification and characterization of five new OP2-related *Myoviridae* bacteriophages infecting different strains of *Xanthomonas oryzae* pv. *oryzae*. *Journal of Plant Pathology* **101(2), 263-273.**

Pandey, S., Bohra, A., Singh, B., Prasad, P. and Kumar, J. (2017). Haplotypic diversity and virulence of *Xanthomonas oryzae* pv. *oryzae* population infecting the first superfine aromatic basmati hybrid. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences* **87(3), 1005-1014.**

Ranjani, P., Gowthami, Y., Gnanamanickam, S. S. and Palani, P. J. M. (2018). Bacteriophages: A new weapon for the control of bacterial blight disease in rice caused by *Xanthomonas oryzae*. *Microbiology Biotechnology Letters* **46(4), 346-359.**

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York. **11, 31.**

Silva, J. B., Storms, Z. and Sauvageau, D. (2016). Host receptors for bacteriophage adsorption. *FEMS Microbiology Letters* **363(4), fnw002.**

Tanji, Y., Shimada, T., Yoichi, M., Miyanaga, K., Hori, K. and Unno, H. (2004). Toward rational control of Escherichia coli O157: H7 by a phage cocktail. *Applied Microbiology and Biotechnology* **64, 270-274.**

Xuan, T., Hoang, H. A. and Tam, L. (2018). Stability and activity of TG25P phage in control of *Aeromonas hydrophila* in striped catfish pond water. *VNUHCM Journal of Science and Technology Development* **21(2), 64-70.**

Yasmin, S., Hafeez, F. Y., Mirza, M. S., Rasul, M., Arshad, H. M. I., Zubair, M. *et al.* (2017). Biocontrol of bacterial leaf blight of rice and profiling of secondary metabolites produced by rhizospheric *Pseudomonas aeruginosa* BRp3. *Frontiers in Microbiology* **8, 1895.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538