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Efficacy of bacteriophage L522 against bacterial leaf blight of rice in Vietnam

Pham D.T. My[1,2], Le T.T. Tien[1,2], Le P. Nga[1,2], To H. Ngoc[1,2], Vo T. Phuc[1,2], Hoang A. Hoang[1,2]*

[1]Department of Biotechnology, 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.

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ABSTRACT

Bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a destructive rice disease in tropical Asian countries. The conventional approaches to control BLB, such as cultural practices and using chemical pesticides remain ineffective, whereas bacteriophage biocontrol is considered as a promising strategy. The current study investigated the capacity of phage L522 to control BLB in rice. First, phage L522 showed high tolerance over a wide range of temperatures (4, 20, 30, 37, and 50°C) and pH (4–11) and was also less affected by ultraviolet (UV)-A and UV-B light. Second, the inhibition time of the phage on the growth of *Xoo* in tryptone soya broth was about 45 h in the *in vitro* test. More interestingly, in the *in vivo* trial on rice plants, the efficacy of phage treatment at a concentration of approximately 10^9 PFU/mL was equivalent to that of a popular commercial pesticide. This study indicated that phage L522 should be a suitable and environmental-friendly alternative to control BLB, especially applicable to soil and climatic conditions in Vietnam.

ARTICLE HIGHLIGHTS

- Phage L522 showed a high stability under various temperatures, pH, and ultraviolet light.
- In the *in vitro* test, phage L522 controlled the growth of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) for approximately 45 h.
- In the *in vivo* trial, phage L522 had an equivalent efficacy against bacterial leaf blight in rice compared to a commercial pesticide.

1. INTRODUCTION

Bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. oryzae (*Xoo*) could lead to enormous yield losses of up to 50% in rice production areas [1]. Most Vietnamese high-quality rice varieties are susceptible to BLB resulting in 15–30% reductions in annual rice yield [2]. In southern Vietnam, BLB often occurs throughout the rainy season (from May to October). The soil-borne pathogens infiltrate natural openings or wounds in plants, leading to their rapid multiplication and widespread dissemination across the cultivation area through wind and heavy rain. The conventional approaches to control BLB, such as cultural practices and using chemical pesticides or biological control agents, remain ineffective, especially during the epiphytic stage [3]. Besides, rampant usage of chemical pesticides in agriculture has raised concerns about adverse effects on the ecosystem and human health in recent decades. In addition to decreasing the efficiency of BLB management, the development of antibiotic resistance in the *Xoo* population may be responsible for antibiotic resistance transmission into non-target microbiota [4,5]. In efforts to find more environmental-friendly alternatives to antibiotics, phage biocontrol has been considered a promising strategy for the control of phytopathogens.

Phage biocontrol against *Xanthomonas* sp. has been studied [6]. The host cells are specifically infected and lysed through the replication cycles of lytic bacteriophages. Therefore, phages are harmless to other non-target microbes in the normal microbiota and nontoxic to eukaryotic cells. Moreover, the observed reduction in virulence of phage-resistant *Xoo* may indirectly enhance the effectiveness of phage biocontrol [7]. Several studies indicated positive results in the inhibitory activity of isolated phages on *Xoo* in laboratory and greenhouse conditions [8,9]. In addition to genome properties [10], phage viability in various influence factors, such as temperature, pH, and ultraviolet (UV) radiation, should be investigated for phage production, storage, and practical application purposes. In a previous study of the genome properties with the NCBI accession number OP948730, phage L522 was considered a safe candidate for phage biocontrol [11]. The current study investigated the phage stability under environmental factors and the *in vivo* efficacy of phage L522 to control BLB disease in rice.

*Corresponding Author:
A. Hoang Hoang,
Department of Biotechnology, Faculty of Chemical Engineering, Ho Chi Minh City University of Technology (HCMUT), 268 Ly Thuong Kiet, District 10, Ho Chi Minh City, Vietnam.
E-mail: hoang.a.hoang @ hcmut.edu.vn

2. MATERIALS AND METHODS

2.1. Phage Stability Test

The differences in phage L522 viability under various physicochemical conditions (temperature, pH, UV-A, and UV-B) were assessed according to the method of Xuan *et al.* [12]. To determine the heat stability, phage preparation was diluted in SM buffer (100 mM NaCl, 10 mM $MgSO_4$, 0.01% gelatin, 50 mM Tris-HCl, pH = 7.5) at approximately 10^7 PFU/mL, then incubated at different temperatures (4, 20, 30, 37, and 50°C) for 1 h. Samples taken at 10-min intervals were titrated using a plaque assay with bacterial strain *Xoo* LA1+. In the pH stability test, either 1 M HCl or 1 M NaOH was used to adjust the pH value of tryptone soya broth (TSB) to obtain solutions with a pH range of 3–11. The phage suspension (~ 10^7 PFU/mL) was added to an equal volume of the adjusted TSB and incubated at 30°C. After 24-h incubation, the phage titer was determined. The effect of UV radiation was also studied at 311 nm (UV-B, model Philips PL-S 9W/01/2P) and 365 nm (UV-A, model Phillips Actinic BL TL-D 15W). Phage stock was diluted in SM buffer to an initial titer of ~ 10^8 PFU/mL. A 2-mL dilution was placed on an Φ-6mm petri dish and exposed to UV radiation from a distance of 30 cm for 1 h. Sampling was carried out every 10 min and then titrated. The experiment was conducted in triplicate.

2.2. Bacterial Challenge Test

To evaluate the inhibitory activity of phage L522 against *Xoo*, the bacterial challenge test was conducted. The culture of *Xoo* strain LA1+ was incubated at 30°C with shaking at 150 rpm in TSB medium. Until the absorbance at 600 nm reached approximately 0.1 (~ 6×10^7 CFU/mL), a volume of the phage suspension was added at a multiplicity of infection (MOI) of 0.01, 0.1, or 1.0. The sample without phage was used as the control. Mixtures were incubated as described above. Sampling was carried out periodically to determine the OD_{600}, which indicated the changes in bacterial growth by phage lysis. The experiment was performed in triplicate.

2.3. Preparation of Phage Suspension for the *In Vivo* Trial

The culture of *Xoo* strain LA1+ was incubated at 30°C with shaking at 200 rpm in TSB medium. Until the absorbance at 600 nm reached approximately 0.1, a volume of the phage stock was added at an MOI of 0.1. After 8 h shaking as above condition, the mixture became clear and was then distributed into 2-mL centrifuge tubes and centrifuged at 9,727 × g for 5 min at 4°C. The supernatant was passed through 0.22-µm filter membranes to obtain the final phage suspension.

2.4. *In Vivo* Trial of Phage in Rice

The *in vivo* efficacy of phage L522 to control BLB was assessed at Southern Research Center for Plant Protection in Long An province, Vietnam. Jasmin 85 rice variety provided by Cuu Long Delta Rice Research Institute was used. After pre-germinated at room temperature for 48 h, 5 seeds were sown in each 17.5 × 12 cm plastic pot containing 1 kg of soil. Then rice seedlings were pruned to 3 plants per pot. After 45 days of sowing, the experiment was conducted in an arrangement of completely randomized design at environmental temperature with three replications per treatment. Rice plants were infected with the *Xoo* L024 from microbial-type culture collection in the laboratory, by wound-inducing and spraying bacterial suspension at a concentration of approximately 10^8 CFU/mL. At day 1 and day 5 after the bacterial infection, four treatments were sprayed phages at different titers such as ~10^6, 10^7, 10^8, 10^9 PFU/mL, respectively. Another treatment was sprayed a popular commercial pesticide in Vietnam (Starner 20WP) according to the manufacturer's instruction. The control was infected plants sprayed with sterile water. The plants were observed daily. On day 7 after the second treatment with phage, disease rate (%) and disease incidence (%) were measured as following equations:

$$\text{The disease rate (\%)} = \frac{\text{Number of diseased leaves}}{\text{Total number of leaves in the treatment}} \times 100$$

$$\text{The disease incidence (\%)} = \frac{9 \times n_9 + 7 \times n_7 + 5 \times n_5 + 3 \times n_3 + 1 \times n_1}{9 \times N} \times 100$$

n_1, n_3, n_5, n_7: Corresponding to the number of leaves whose diseased area was <1%, 5%, 25%, 50%; n9: The number of leaves whose diseased area was >50%. N: total number of leaves in the treatment.

Curative ratio (%) was calculated as following equation:

$$\text{Curative ratio (\%)} = \frac{\text{DI of infected plant} - \text{DI of treated plant}}{\text{DI of infected plant}} \times 100$$

DI stands for Disease Incidence. The treated plant in this equation indicated plants treated with phage or pesticide in each test. The above assessment was based on the national technical regulation on surveillance methods of rice pests [13]. Statistical analysis was performed using one-way analysis of variance by IBM SPSS Statistics 20 Software, and the significant differences between means were determined by Duncan's multiple range test at $P \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Effect of Temperature, pH, and UV Radiation on the Viability of Phage

As shown in Figure 1a, the temperature stability of phage L522 was stable at all tested temperatures during 1-h incubation. Even at 50°C, the phage concentration was similar to the initial value ($P > 0.05$). The same results were found with several published *Xanthomonas* phages [6]. In addition, the titer of the phage remained unchanged over a wide pH range of 4 to 11 as presented in Figure 1b. It is clarified that phage L522 is more resistant to acidic environment than phage ϕXOF4, the titer of which was affected by pH below 6 after 1-h exposure [14]. The phage titer only sharply reduced at pH 3. While temperature and environment acidification are the main factors limiting phage activity [15], the high tolerance of phage L522 contributed information to devise suitable application strategies in rice farming.

Figure 1c presents that the phage viability was gradually less affected by UV-A and UV-B light during 1-h exposure. Viable phage counts slightly declined and reached by ~ 0.5 log 10 units at the end of the experiment under UV-B. However, no significant changes in phage titer were recorded under UV-A radiation. Natural sunlight UV level (UV-A and UV-B) is one of the stability issues when applied in both greenhouse and field conditions. Efforts are being made to prolong the viability of phages on the rice leaf surface, including preventing daylight exposure or improving phage formulation [16].

3.2. Inactivation of *Xoo* in Broth by Phage

The inhibition of phage L522 on *Xoo* growth in TSB medium was evaluated at three MOI (0.01, 0.1, 1.0). From the initial concentration (OD_{600} ~ 0.1), the growth of host culture was inhibited post-infection

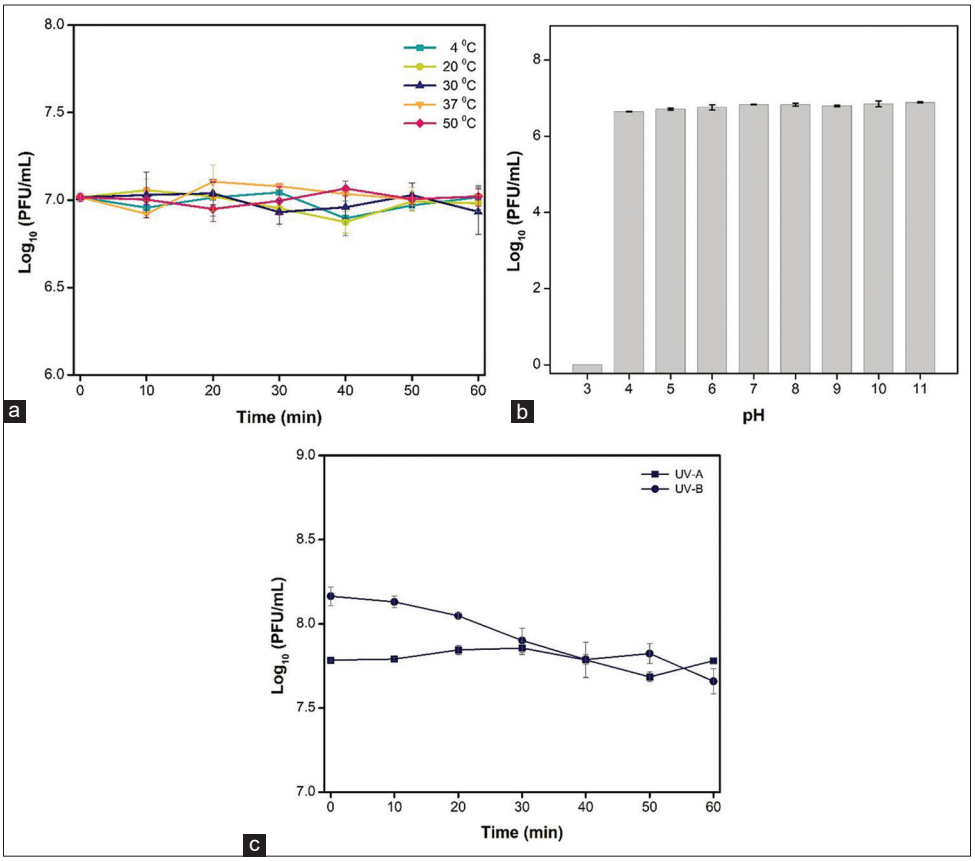

Figure 1: Stability of the phage L522 under various conditions including temperatures (a), pHs (b), and ultraviolet (UV)-A and UV-B exposure (c). Error bars indicate 95% confidence intervals for the averaged values (n = 3).

(p.i.) by phage and the mixture became clear at 6 h p.i. Figure 2 shows that the host OD_{600} began to decrease at different times depending on the MOI. In detail, while the OD_{600} of the mixture with MOI of 0.1 and 1.0 started to decline at 2 h p.i, the OD_{600} of host culture with an MOI of 0.01 remained at about 0.1 until 4 h p.i. It is proved that the higher phage concentration resulted in a faster inhibitory effect at the beginning. Growth inhibition was maintained for approximately 45 h by phage infection, whereas the control culture without phage grew sharply and entered the stationary phase at around 30 h (OD_{600} ~ 2). After 45 h p.i, there were gradual increases of OD_{600} in all three phage treatments disregarding MOI that reflected the growth of phage-resistant bacteria. The OD_{600} returned to ~ 0.1 after 55 h. There was no significant difference in the inhibition time among different MOIs. The results of the *in vitro* test revealed that L522 phage is reasonable to be studied further in the *in vivo* trial.

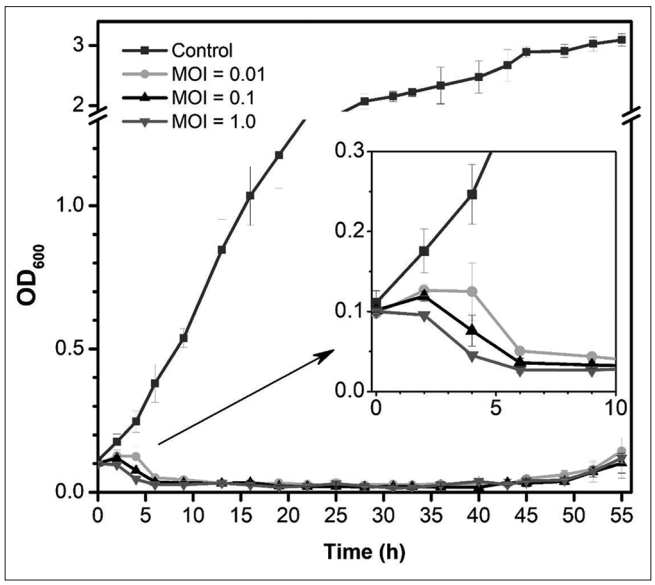

Figure 2: Changes in optical density at 600 nm during inactivation of *Xoo* LA1+ by L522 in tryptone soya broth medium at an multiplicity of infection of 0.01, 0.1, or 1.0. The sample without phage was used as the control. Error bars indicate 95% confidence intervals for the averaged values (n = 3).

3.3. *In Vivo* Efficacy of Phage in Rice

The concentration of phage suspension prepared for this experiment was approximately 10^9 PFU/mL. This phage preparation was then diluted to desired concentrations. In the *planta* pot experiment, treatments at four phage titers (10^6, 10^7, 10^8, 10^9 PFU/mL) and a commercial pesticide (Starner 20WP), were applied twice at day 1 and day 5 after the bacterial inoculation. On day 7 after the second treatment, the tests showed significant differences [Figure 3 and Table 1]. Compared to the control, rice leaves treated with phage L522 and the pesticide were obviously less damaged. Table 1 shows that the lowest disease rate and disease incidence of BLB occurred when applying the phage concentration of 10^9 PFU/mL. With the highest phage titer, the disease rate decreased by approximately 9%, whereas that of the pesticide treatment did not significantly differ from the control plants. Moreover, the efficacy of these two treatments (the phage

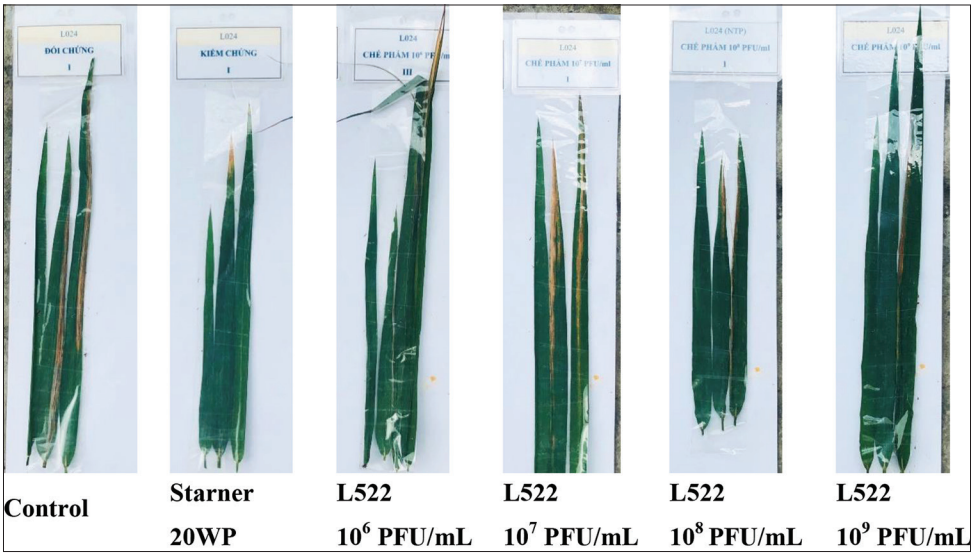

Figure 3: Differences in the lesion length of rice leaves by treatment with Starner 20WP and phage at various concentrations (10^6, 10^7, 10^8, 10^9 PFU/mL) at day 12 after *Xoo* L024 infection. In the control, bacterial infected plants were sprayed with sterile water.

Table 1: *In vivo* efficacy to control bacterial leaf blight in rice by Starner 20WP treatment and phage treatments at various concentrations (10^6, 10^7, 10^8, 10^9 PFU/mL) at day 12 after *Xoo* L024 infection.

Phage concentration	Disease rate (%)	Disease incidence (%)	Curative ratio (%)
10^6	28.0[a]	9.44[b]	18.2[c]
10^7	20.8[bc]	8.59[b]	25.4[c]
10^8	23.7[b]	7.54[c]	34.5[b]
10^9	20.5[c]	6.38[d]	44.7[a]
Starner 20WP	27.1[a]	6.25[d]	45.7[a]
Control	29.3[a]	11.5[a]	na
cv (%)	6.48	6.57	12.2

The same letters on the same columns indicate numbers are not significant according to Duncan's multiple range test at 0.05 level.

concentration of 10^9 PFU/mL and the pesticide) in reducing the disease incidence was equivalent. The disease severity and extent declined to one-half of the control. These results may induce the similarity in the curative ratio of phage preparation at 10^9 PFU/mL (44.7%) and the pesticide application (45.7%). Further, the *in vivo* efficacy to control BLB decreased at lower phage concentrations (10^6, 10^7, 10^8 PFU/mL). As shown in Table 1, the phage titer at 10^8 PFU/mL is considered as a second option of treatment following 10^9 PFU/mL, when its curative ratio was obviously higher than other phage treatments (10^6 and 10^7 PFU/mL). The active chemical of Starner 20WP is oxolinic acid, one of the antibiotics most commonly used against plant pathogen bacteria worldwide [17]. However, the gradual decrease in oxolinic acid efficacy due to resistant strains was recorded during 2009 – 2014 in Israel [18]. The antibiotic resistance could spread to the surrounding environment via plasmid-mediated quinolone resistance genes [19]. As comparable efficacy to Starner 20WP, the phage L522 application is suggested as a more environmental-friendly alternative to control BLB.

4. CONCLUSION

Phage biocontrol is a potential solution in the fight against plant diseases, especially BLB in rice. Our research contributed a profile to the existing *Xoo* phage collection for practical applications under different soil and climatic conditions. The phage stability, *in vitro*, and *in vivo* control efficacy of phage L522 were evaluated. Besides the safety reported in the previous paper, the results of this study suggest that phage L522 has an equivalent efficacy in control BLB in rice to the commercial pesticide Starner 20WP. Therefore, phage L522 is a promising candidate for BLB management in rice farming.

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6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

10. PUBLISHER'S NOTE

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