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Production of Bacteriophage L522 for Management of Bacterial Leaf Blight on Rice in Field Trial

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Rice cultivation, particularly in tropical Asian countries, has faced a serious challenge from bacterial leaf blight (BLB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Bacteriophage biocontrol provides a potential solution for sustainable BLB management, offering enhanced safety and environmental sustainability compared to antibiotics. The first objective of this study was to investigate suitable culture media and incubation temperature for enhancing the growth rate of the host *Xanthomonas oryzae* pv. *oryzae* LA1+. The optimal time for adding phage into the bacterial culture and the sufficient infection time were also identified. Tryptone Soya Broth was selected as the nutrient medium for phage propagation. Six hours post-bacterial inoculation into fresh medium, bacteriophage L522 was added, and then the mixture was shaken at 150 rpm under the temperature of 28 – 30 °C for 6 h. These conditions resulted in the highest final phage titer of 9.5 log10 PFU/mL ($p < 0.001$). The second objective was to evaluate the efficacy of L522 to manage BLB in a field trial. Phage treatment showed the same effectiveness as the popular pesticide Starner 20WP in controlling BLB in the rice field ($p = 0.391$). This study presented a strategy for producing phage L522 and indicated the promising efficacy of phage biocontrol of BLB in the prevalence of antibiotic-resistant *Xoo* in rice.

1. Introduction

Bacteriophages are viruses that lyse bacteria with highly specific infection. Many researchers have studied bacteriophage as a weapon against pathogens in agriculture since the 20th century. In 1924, Mallmann and Hemstreet used the filtrate containing phage from decomposed cabbage to prevent the spread of *Xanthomonas campestris* pv. *campestris* causing black rot in cabbage (Mallmann and Hemstreet, 1924). Since the beginning of the 21st century, due to the prevalence of antibiotic-resistant bacteria, research on phages has increased, becoming a promising solution. In the list of 10 common plant pathogenic bacteria (Mansfield et al., 2012), *Pectobacterium*, *Ralstonia*, *Pseudomonas* and *Xanthomonas* are groups of bacteria with many scientific publications and patents related to phage-based solutions. Surveying 21 patents and 137 scientific articles, the publications focused on 4 main topics: (1) phage isolation and characterization, (2) phage cocktail, (3) phage production, and (4) strategies in disease prevention and treatment and effectiveness evaluation (Holtappels et al., 2019).

Bacterial leaf blight (BLB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), threatens rice cultivation worldwide, with a significant impact on tropical Asian countries where rice is the main food staple (Verdier et al., 2012). The study by Kuo et al. (1969) was the first research on bacteriophages, isolated from water in rice fields, to control *Xoo*. The lytic activity of phage Xf was 100 % when incubated with bacteria and remained stable after 7 days. For practical application, studies in phage production are required to establish a procedure that maximizes phage titer and productivity. Factors relating to the host, such as physiological state, metabolic activity, and growth rate (affected by growth conditions), influence phage replication. The multiplicity of infection (MOI) and initial bacterial concentration must also be considered (Santos et al., 2014). Furthermore, the optimal harvest time can be determined to obtain the maximum phage titer in an efficient and time-saving process (Tanir et al., 2021). Several studies report the efficacy of *Xoo* phage in controlling the BLB pathogen both *in vitro* and *in vivo*. For instance, phage vB_XooS_NR08 maintained a 99.95 % reduction in bacterial count for 48 h in Peptone Sucrose Broth, and decreased the disease severity by 79.27 % compared to the untreated control in a

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pot assay (Jain et al., 2023). Additionally, Liu et al. (2023) demonstrated that a phage cocktail of pXoo2106 and pXoo2107 inhibited *Xoo* growth for over 48 h and effectively reduced pathogen concentration in the *in vivo* test. However, there are no studies about optimizing conditions for *Xoo* phage propagation.

Phage L522 was identified as a safe candidate for phage biocontrol due to the absence of virulence and antibiotic-resistance genes (My et al., 2023). The significant efficacy of phage L522 in managing BLB was demonstrated by My et al. (2024) in the *in vivo* trial with pots of rice plants. However, a field trial is an essential assessment before practical application. Additionally, research into the production of phage L522 is necessary to facilitate the availability of phage preparations for large cultivated areas. In this study, nutrient medium and incubation temperature were optimized to enhance the growth rate of the bacterial strain *Xanthomonas oryzae* pv. *oryzae* LA1+. Furthermore, the optimal time points for initiating and harvesting phage infection were determined to maximize the titer of phage L522. Finally, a field trial was conducted to assess the efficacy of phage L522 in controlling bacterial leaf blight.

2. Materials and Methods

2.1 Selection of culture media and effect of temperature on *Xoo* growth

The strain *Xanthomonas oryzae* pv. *oryzae* LA1+, isolated in Long An province, was selected for phage production. The growth of *Xoo* LA1+ in two different kinds of culture media, including Tryptone Soya Broth (HiMedia, Soya peptone 3 g/L, Tryptone 17 g/L, NaCl 5 g/L, K_2HPO_4 2.5 g/L, Glucose 2.5 g/L, pH 7.3 ± 0.2) and Nutrient Broth (HiMedia, Peptone 5 g/L, Yeast extract 1.5 g/L, HM peptone B 3 g/L, NaCl 5 g/L, pH 7.4 ± 0.2), was compared. Initially, the overnight culture of bacteria was added to the tested media with an OD_{600} ~ 0.1 (~ 10^8 CFU/mL), then incubated at 30 °C with shaking at 150 rpm. Samples were taken every 1 h and diluted in 0.85 % NaCl to determine the bacterial population using the plate count method.

To investigate the effect of temperature on *Xoo* growth, experiments were conducted in the appropriate media based on the results of the above experiment at different incubation temperatures (26, 28, 30, 32, 34, and 36 °C) with shaking at 150 rpm. Beginning with an OD_{600} ~ 0.1, during 9-h incubation, samples were taken every 3 h to determine the bacterial population using the plate count method. The number of surviving bacterial cells was recorded to evaluate the impact of each temperature on the growth rate of *Xoo* throughout the experiment.

2.2 Influence of infection point and endpoint on phage titer

The experiment was designed to evaluate the optimal time point for adding phage into bacterial culture and to estimate the infection time required to achieve the highest phage titer. Firstly, the overnight culture of *Xoo* LA1+ was inoculated into the selected media (5 % v/v), then adjusted to OD_{600} ~ 0.07, and incubated under the appropriate temperature with shaking at 150 rpm. Secondly, a volume of phage L522 (10^9 PFU/mL) was added to the host growing culture (1 % v/v) at 0, 2, 4, and 6 h after the initial inoculation. At each infection point experiment, samples were taken after 6 h and 8 h of infection, then the phage titer was determined by the plaque assay method (Hoang et al., 2018). The host culture without phage infection was used to monitor the growth of *Xoo* LA1+. The actual MOI (Multiplicity of infection) of the individual experiment of infection point was also estimated.

2.3 Production of phage suspension for the field trial

The propagation of phage L522 was carried out under the conditions selected from the above experiments. To remove host cells, the mixture was centrifuged at 4,000 rpm for 10 min, followed by filtering the supernatant using WaterSep cross-flow filtration with a 0.45-µm filter membrane. The filtration process was repeated by using a vacuum filtration system with a 0.45-µm filter membrane in the microbiological safety cabinet. The final cell-free phage suspension was obtained and stored at 4 °C.

2.4 The efficacy assessment of phage in the field trial

The efficacy of phage L522 in the field trial to control BLB was assessed on Jasmin 85 rice variety in Long An province, Vietnam. The field experiment featured a RCBD (Randomized complete block design) with six treatments replicated three times: (1) combined strategy of pre-treatment before bacterial inoculation and treatment by phage suspension at 10^7 PFU/mL and (2) 10^8 PFU/mL, (3) treatment by phage suspension at 10^7 PFU/mL and (4) 10^8 PFU/mL after bacterial inoculation, (5) a popular commercial pesticide in Vietnam (Starner 20WP), and (6) a no-phage control. A total of 18 rice planting plots were established, with each plot occupying an area of 50 m^2 (5 × 10 m) with 0.5 m spacing between plot edges. After ~ 50 days of sowing, all of the planting plots were artificially wounded and spray-inoculated with *Xoo* L024 (prepared from the laboratory culture collection) at a concentration of approximately 10^8 CFU/mL to induce the bacterial leaf blight. For the combined strategy, phage treatment areas were sprayed with phage suspensions in the late afternoon the day before inoculation, then repeated on day 1 and 5. In the experiment (3) and (4), phage suspensions were sprayed on

day 1 and 5 after bacterial inoculation. Starner 20WP treatment in the experiment (5) was conducted according to the manufacturer's instructions post-inoculation and the no-phage control (6) was sprayed with sterile water on the same days as phage treatments.

After 12 days of bacterial inoculation, sampling was conducted to assess the severity of bacterial leaf blight. Within each plot, five sampling points were randomly selected along two diagonal lines, ensuring a minimum distance of 0.5 m from the plot edge. At each sampling point, a 0.2 m^2 (0.4 × 0.5 m) quadrat was used to determine the disease rate (%) and the disease incidence (%) based on the national technical regulation on surveillance method of rice pests (PPD, 2014), then the curative ratio (%) was also calculated (My et al., 2024).

2.5 Statistical analysis

The data were statistically analysed using one-way analysis of variance (ANOVA) in IBM SPSS Statistics 20 software with Duncan's multiple range test applied to compare means at a significance level of $p \leq 0.05$.

3. Results and Discussion

3.1 Suitable nutrient medium and temperature of incubation for *Xoo* growth

To compare the *Xoo* growth in Tryptone Soya Broth (TSB) and Nutrient Broth (NB), the values of bacterial density measured during 9-h experiments in these two media were plotted. According to the Figure 1a, the growth curves demonstrate similar growth patterns in both TSB and NB. Following a lag phase of approximately 5 h, the bacterial densities increased from the initial value of ~10^8 CFU/mL to the highest population of ~10^9 CFU/mL after 9 h. Tryptone Soya Broth (TSB) was selected for next experiments.

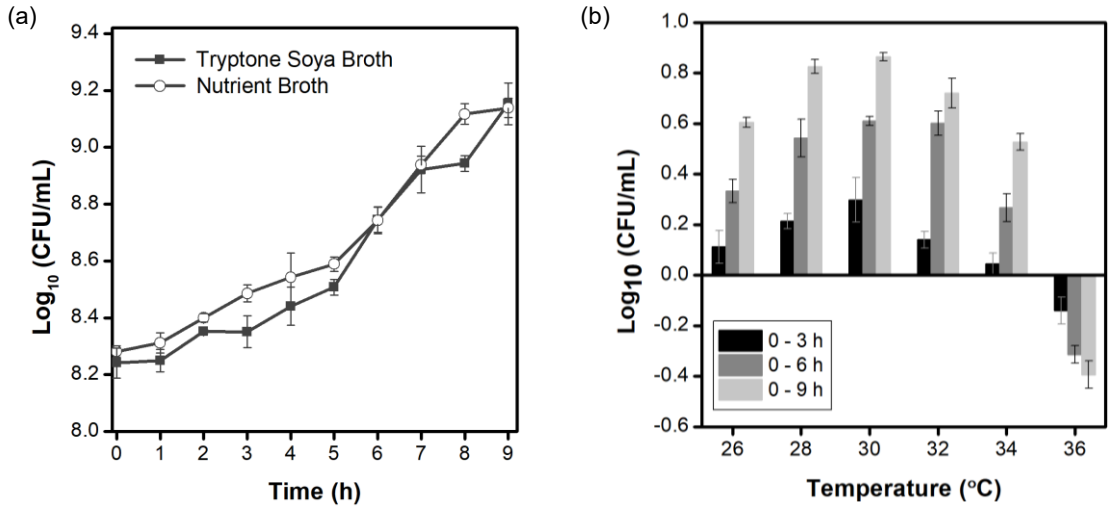

Figure 1: (a) The growth of Xanthomonas oryzae pv. oryzae LA1+ in (a) Tryptone Soya Broth and Nutrient Broth, and (b) under different temperatures.

The maximum temperature for *Xanthomonas sp.* growth varies from 27.5 to 39 °C (Saddler and Bradbury, 2005). Therefore, to balance optimal growth conditions with practical considerations for production facilities, the experiment was set up across a wide range of temperatures. The optimal temperature range for *Xoo* growth was determined in Tryptone Soya Broth. Figure 1b shows the increase of bacterial density after 3, 6, and 9 h post-inoculation (hpi) across a temperature gradient of 26, 28, 30, 32, 34, and 36 °C. At 3 hpi, bacterial cells increased slightly by ~ 0.1 log10 CFU/mL across all temperatures except 36 °C. However, at 6 hpi, compared to the initial value, bacterial counts increased significantly by ~ 0.5 log10 CFU/mL at 28, 30, and 32 °C. Over the 9-h incubation, the growth rate of *Xoo* reached a maximum of ~ 0.8 log10 CFU/mL at 28 and 30 °C. Besides, bacterial survival was inhibited at 36 °C, as the density had decreased noticeably since 3 hpi. Therefore, the suitable temperature range for *Xoo* growth in Tryptone Soya Broth is 28 – 30 °C.

3.2 Influence of infection point and endpoint on phage titer

Based on the conclusions from the above tests, the experiment was carried out in Tryptone Soya Broth at 28 – 30 °C to identify the optimal time for adding phage into the bacterial culture and the sufficient infection time.

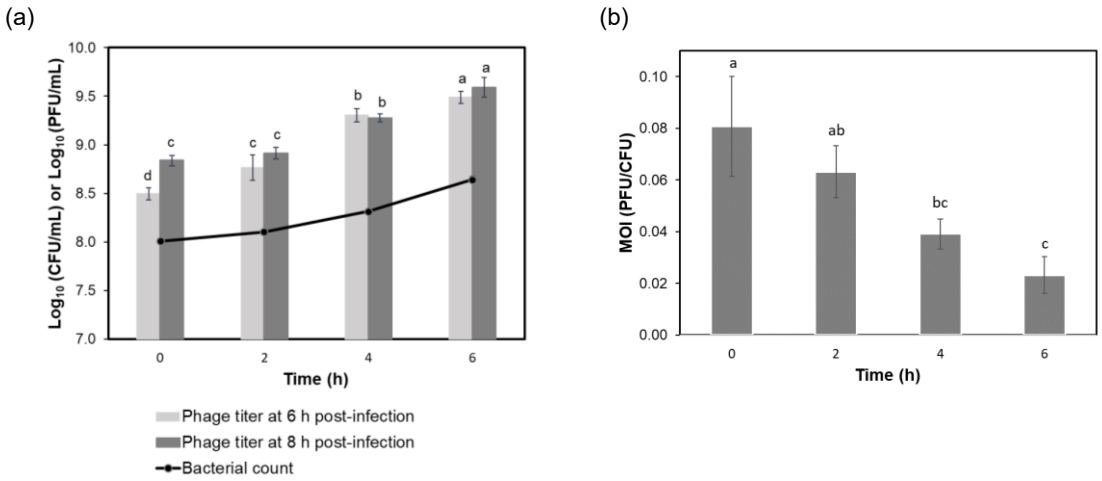

Figure 2: (a) Bacterial growth and phage titer variations at 6 and 8 h post-infection, with varying initial infection times and (b) the actual MOI.

As shown in Figure 2a, the later the phage was added, the higher the final phage titer was obtained. Specifically, the lowest phage density of 8.5 log10 PFU/mL was recorded when phage L522 was added immediately after inoculation, whereas the highest phage density reached 9.5 log10 PFU/mL when phage was added at 6 h post-inoculation (hpi) ($p < 0.001$). It was demonstrated that the physiological state of host cells could affect phage reproduction (Nabergoj et al., 2018). From 4 hpi, the rapid growth rate of *Xoo* LA1+ was observed, indicating the high proportion of good-quality cells that promoted the production of phage L522. Besides, the batch model for producing *Salmonella* phage presented that the initial phage concentration, initial bacterial concentration (used to calculate the actual MOI value), phage adsorption rate, bacterial growth rate, and infection time are essential parameters in phage production (Santos et al., 2014). Depending on different objectives, these parameters will be adjusted. According to Figure 2b, the actual MOI decreased when the infection point was delayed; however, the lowest MOI of 0.02 still resulted in the highest phage titer. Therefore, in this study, the physiological state of host cells influenced the phage production more than the MOI. A similar result was reported by Duyen et al. (2022) that MOI in a range of 0.01 - 1.0 produced similar phage yields. Furthermore, there was no significant difference in the final phage counts between the infection times of 6 h and 8 h ($p = 0.207$). Finally, the highest phage titer was achieved by adding phage L522 suspension into the *Xoo* LA1+ culture at 6 h post-inoculation, followed by a 6-h infection period.

3.3 Efficacy of phage in field trial

To assess the efficacy of phage in controlling BLB, the experiment was designed in a paddy field with two concentrations of phage suspension (Figure 3).

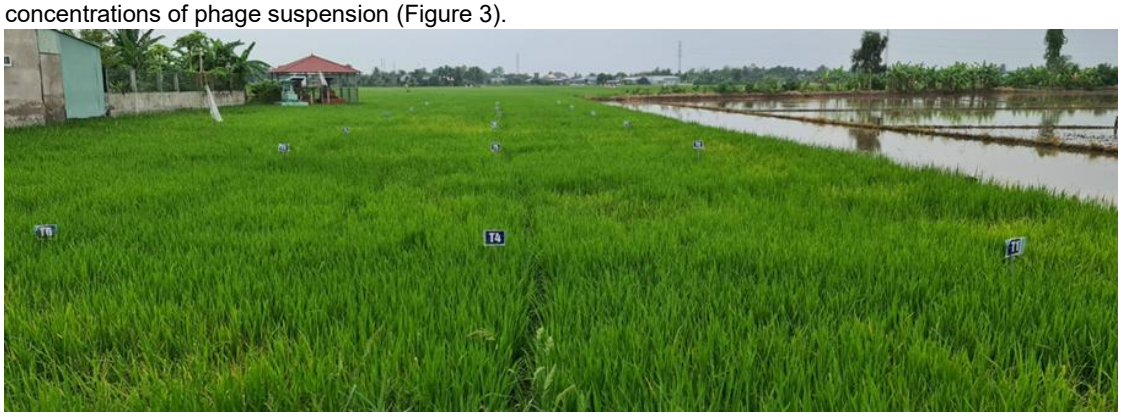

Figure 3: Field trial in Long An province, Vietnam.

Table 1: Efficacy of phage treatments in controlling bacterial leaf blight on day 12 after Xoo L024 inoculation in field trial

Phage concentration	Disease rate (%)	Disease incidence (%)	Curative ratio (%)
10^7 (pre-inoculation)	18.9[a]	3.72[b]	14.2[b]
10^8 (pre-inoculation)	19.4[a]	2.78[c]	36.0[a]
10^7 (post-inoculation)	20.2[a]	3.50[b]	19.3[b]
10^8 (post-inoculation)	18.8[a]	2.76[c]	36.5[a]
Starner 20WP	15.6[b]	2.59[c]	40.3[a]
Control	20.7[a]	4.34[a]	na
cv (%)	6.97	6.02	18.9

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

Table 1 presents index values for the BLB severity, including the disease rate and the disease incidence, and the curative ratio of phage treatments and Starner 20WP treatment. Regarding the disease rate, pesticide treatment showed the most effectiveness in controlling the percentage of infected plants in the assessed area, while there is no significant difference between phage treatments and the control ($p = 0.129$). However, the disease incidence values of both phage treatments and Starner 20WP were significantly lower than the control. In particular, the severity of BLB on plants treated by phage at a concentration of 10^8 PFU/mL was effectively controlled, the same as the pesticide ($p = 0.281$). The disease incidence when using the highest phage concentration and Starner 20WP was 2.76 and 2.59, respectively, whereas the disease incidence of the control was 4.34. Moreover, the phage's efficiency in controlling BLB was similar for both pre- and post-inoculation treatments while using the same phage concentration. The curative ratio of the treatment using the phage concentration of 10^7 PFU/mL was 14.2 % and 19.3 % respectively for pre- and post-inoculation treatments ($p = 0.290$).

Consequently, the best curative ratio of phage treatment was achieved using the phage concentration of 10^8 PFU/mL, showing similar efficacy to Starner 20WP ($p = 0.391$). Specifically, the phage's efficiency in managing BLB was 36.0 – 36.8 %, while the efficiency of Starner 20WP was 40.3 %. The experiment demonstrated that phage biocontrol could be effective as a pesticide but safer and environmentally friendly. Moreover, the prevalence of antibiotic resistance is widely reported in plant pathogens, notably in rice with cases of *Burkholderia glumae* resistant to oxolinic acid, the active component of Starner 20WP (Maeda et al., 2004) and *Xanthomonas oryzae* resistant to the popular zhongshengmycin (Wang et al., 2021), even showing resistance to the novel bactericide fubianezuofeng (Yi et al., 2020). Consequently, the effectiveness of antibiotics in controlling bacterial diseases is diminished. In contrast, while phage resistance naturally exists, phage banks allow for the prompt selection of phages to address the emergence of phage-resistant bacteria.

4. Conclusion

Bacteriophage biocontrol is a promising treatment to control phytopathogens, addressing concerns about the disadvantages of antibiotic usage. Phage-based products are considered as a potential alternative for managing rice diseases, particularly bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae*, when antibiotic resistance is widely reported even with a novel bactericide. For practical application, optimizing conditions for phage propagation in the upstream process is essential for commercializing phage preparation. Conditions of phage L522 production were investigated to obtain high productivity. Using the strain *Xoo* LA1+, phage propagation should be carried out in Tryptone Soya Broth at a temperature of 28 – 30°C. The ideal infection time was determined at 6 h post-bacterial inoculation, followed by 6 h shaking at 150 rpm to achieve the highest final phage titer of 9.5 log10 PFU/mL. Phage suspensions were applied in a field trial and demonstrated high efficacy in controlling bacterial leaf blight, compared with the popular pesticide Starner 20WP. The promising results in the field trial indicated the practical viability of phage L522 as a targeted and environmentally friendly alternative to traditional pesticides to control BLB. This highlights the potential of bacteriophages as an effective and safe biocontrol agent.

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