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Applications of bacteriophages in controlling rice bacterial grain rot caused by *Burkholderia glumae*

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ABSTRACT

The study on the procedure of applicating bacteriophage (or phage) to prevent rice bacterial grain rot caused by Burkholderia glumae was conducted in the greenhouse conditions. The first experiment investigated the effect of different phage titers (i.e. 10^5 PFU/mL, 10^6 PFU/mL, 10^7 PFU/mL, 10^8 PFU/mL) in controlling bacterial grain rot of rice. The results showed that all four titers gave disease reduction with different levels, among these were the titer of 10^8PFU/mL expressed highest efficacy in disease reduction with the lowest percentage of infected grains compared to the rest treatments. The second experiment examined the effect of phage application times (i.e. spraying phage at 2 hours before pathogen inoculation, 2 hours before and 5 days after pathogen inoculation, and 5 days after pathogen inoculation) in suppressing bacterial grain rot disease. The results found that two treatments (i.e. one time spraying at 2 hours before pathogen inoculation, and two times spraying at 2 hours before and 5 days after pathogen inoculation) expressed high efficacy in reduction of grain rot disease through percentage of infected grains and improved yield parameter regarding rate of filled grains.

1. INTRODUCTION

Bacterial grain rot caused by *Burkholderia glumae* is one of the most important diseases on rice (Karki, 2010; Ham et al., 2011; Li et al., 2016; Li et al., 2017; Hasibuan et al., 2018). The bacterium causes infection with seedling rot symptom at seedling stage, panicle blight, and grain rot at flowering stage (Cho et al., 2007; Ham et al., 2011; Gonzalez Beaudion, 2014). The disease has been recorded as one of the serious plant diseases in several countries of Asia, Middle America, Southern America, Southern Africa and has been caused yield loss up to 75% in several states in the USA and more than 40% crop yield losses in Panama (Hasibuan et al., 2018). Chemical use for controlling the disease did not obtain high efficacy based on development of resistant strains (Karki, 2010). Bacteriophage (or phage) therapy is currently considered as a potential method of fighting plant bacterial diseases (Jones et al., 2007). In previous study, lytic phage ΦBurAG58 isolated from (*Burkholderia glumae*) An Giang province that included in this experiment (Doan et al., 2018) was selected as the best promising phage in controlling bacterial grain rot caused by *B. glumae* in *in vitro* and the greenhouse conditions. In this study, the titers and application times of phage ΦBurAG58 were tested for its potential control against bacterial grain rot caused

by *B. glumae* in the greenhouse conditions. This expected result will contribute to further study on phage therapy in suppressing this disease under field conditions.

2. MATERIALS AND METHODS

– **Bacterial strain and culture:** The virulent bacterial strain of *B. glumae* BurDT46 used in this study was isolated from Dong Thap province (Doan et al., 2020). The strain was cultured on King's B medium (20 g peptone, 1.5 g $MgSO_4$. $7H_2O$, 1.5 g K_2HPO_4. $2H_2O$, 15 mL glycerol, 20 g agar, 1000 mL sterile water, pH 7.0-7.2), and incubated 2 days at room temperature. The cultured bacteria were suspended in sterile distilled water, and the suspension was adjusted to OD_{600nm} = 0.3 (corresponded 9 x 10^8 CFU/mL).

– **Preparation of bacteriophages**: The lytic phage ΦBurAG58 isolated from An Giang Province was used in this experiment (Doan et al., 2018). The phage was cultured 1 day at room temperature on King's B medium with 0.8% agar. The cultured phage was suspended in sterile distilled water, then determined phage titer by dilution and plating method. The phage suspension was adjusted to four titers (i.e.10^5 PFU/mL, 10^6 PFU/mL, 10^7 PFU/mL and 10^8 PFU/mL).

– **Preparation of rice plant**: The certificated seeds of OM 4900 variety were used in the experiment. The seeds were surface - sterilized with sodium chloride 15% for 30 minutes. After disinfection, all the seeds were rinsed with sterilized water and then incubated for 48 hours to stimulate the seed germination. The germinated seeds were sowed in a plastic pot (the pot size: 0.049 m^2) containing 7 kg of sterilized soil with the rate of 10 seedlings per pot. Fertilizer was equally added in each pot following the formula of 120 N - 40 P_2O_5 - 50 K_2O kg/ha (Nguyen, 2008).

2.1. Evaluation of phage titers for controlling rice bacterial grain rot in the greenhouse conditions

The experiment was arranged using a completely randomized design (CRD), with five treatments (i.e. applying phage titers: 10^5 PFU/mL, 10^6 PFU/mL, 10^7 PFU/mL, 10^8 PFU/mL, and untreated control), and four replications. Phage titers were sprayed on the flowers (50 mL phage suspension/ pot) at heading stage (55 days after sowing) and flowering stage (60 days after sowing) starting at two hours post inoculation of *B. glumae* BurDT46 suspension with OD_{600nm} = 0.3 (50 mL/pot). The treated pots and untreated control were placed in the greenhouse conditions. The percentage of infected grains were evaluated at 5-days intervals after pathogen inoculation according to the proportion of infected grains from ten panicles per pot in which its calculation was followed the formula: percentages of infected grains = (Number of infected grains x 100/ total number of grains in ten panicles per pot) (Pedraza *et al*., 2018). Disease incidence data from all observation dates was converted to the area under the disease progress curve (AUDPC) (Shanner & Finney, 1977). Yield parameter which included the rate of filled grain was obtained per pot.

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

Where,

Y_i = disease index at time t_i

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

t_i = time when disease index was Y_i

t_{i+1} = time

2.2. Investigation of phage application times for controlling bacterial grain rot in the greenhouse conditions

The experiment was followed a CRD with 4 replications of four treatments in which phage suspensions were sprayed at: (1) 2 hours before pathogen inoculation, (2) 5 days after pathogen inoculation, (3) 2 hours before and 5 days after pathogen inoculation, and (4) untreated control. Disease assessment was recorded as described in the previous experiment (2.1).

2.3. Data analysis

The recorded data on percentage of infected grains, AUDPC and rate of filled grains were subjected to variance at $p \geq 0.05$ and the means were compared with the Duncan Test, using the statistical computer package program MstatC software.

3. RESULTS AND DISCUSSION

3.1. Effect of phage titers application in controlling rice bacterial grain rot in the greenhouse conditions

The control efficacy of phage titers against bacterial grain rot disease was determined by percentage of infected grains from 5-15 days after pathogen inoculation (dai) indicated that all four treatments of applying ΦBurAG58 (10^5 PFU/mL, 10^6 PFU/mL, 10^7 PFU/mL, 10^8 PFU/mL) showed significantly

lower percentage of infected grains than the control. In which the titer of 10^8 PFU/mL was the lowest percentage of *infected grains* and significant difference compared to the others. In addition, all four titers also expressed lower AUDPC from 103 - 409, significantly lower than the control with AUDPC of 725, and treatment 10^8 PFU/mL showed lowest value of AUDPC compared to other phage treatments. Yield parameters regarding rate of filled grains from all phage treatments expressed significantly higher than the control. Specifically, the highest percentage of filled grains was obtained from the treatment of ΦBurAG58 (10^8 PFU/mL) (Table 1).

Table 1. Effect of different phage titers application on percentage of infected grains and rate of filled grains.

Treatments	The percentage of infected grains (%)			AUDPC	Rate of filled grains (%)
	5 dai	10 dai	15 dai		
Control	6.40 [a]	47.0 [a]	53.7 [a]	725[a]	15.3[d]
10^5 PFU/mL	5.11[a]	27.5 [b]	33.2 [b]	409 [b]	48.5 [c]
10^6 PFU/mL	6.00 [a]	17.7 [c]	27.3 [c]	322 [c]	52.1 [bc]
10^7 PFU/mL	7.13 [a]	14.3 [c]	23.1 [c]	278 [c]	63.4 [b]
10^8 PFU/mL	0.00 [b]	7.89 [d]	8.38 [d]	103 [d]	76.5 [a]

Values in the same column with the same letter are not significantly different (p>0.05). dai: days after inoculation.

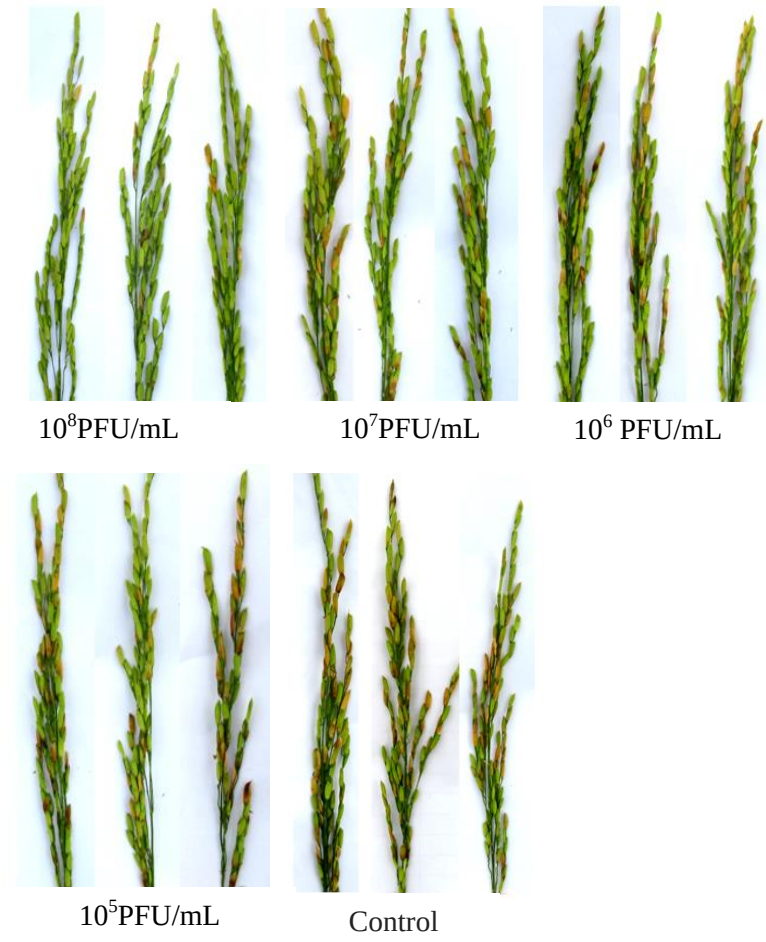

Figure 1. Effect of different titers of phageΦBurAG58 suspension in controlling bacterial grain rot of rice at 10 days after pathogen inoculation

In general, the phage titer 10^8 PFU/mL was the most effective treatment in reducing disease through lowest percentage of infected grains, AUDPC and highest rate of filled grains. This result indicated the high phage titer relevance to high disease protection. Obviously, high phage density will enhance absorption frequency between phage and bacteria that lead to higher disease reduction. Obradovic et al. (2004) reported that phage mixture applied at 10^6 PFU/mL or 10^8 PFU/mL density provided similar levels of control of bacterial spot to tomatoes inoculated with 10^8 PFU/mL of *Xanthomonas perforans*, but at 10^4 PFU/mL was ineffective. Summary, this study concluded that four phage titers from 10^5 to 10^8 PFU/mL showed reduced grain rot disease and higher titers will express more efficiently in disease reduction (Figure 1).

3.2. Effect of phage application times for controlling rice bacterial grain rot in the greenhouse conditions

The data in Table 2 showed that percentage of infected grains from all three ways of phage application were significant lower than untreated control in which treatments of T1 (with spraying phage at 2 hours before pathogen inoculation) and T2 (with spraying phage at 2 hours before pathogen inoculation and 5 days after pathogen inoculation) showed better disease control than T3 (with spraying phage at 5 days after inoculation). Yield parameter as measured by rate of filled grains from all three ways of phage application were significant higher than the control in which phage application before pathogen inoculation (T1) and phage application twice before and after pathogen inoculation (T2) showed higher percentage of filled grains than phage application after pathogen inoculation (T3). The result indicated that treatment of phage before the bacterial (T1) has the effect of preventing bacteria from infecting in the plant. The treatment of phage after inoculation (T3) was less effective because the bacteria had infected the plant, so the treatment ability was lower. Therefore, phage application twice before and after pathogen inoculation (T2) was due to the effect of phage before bacterial inoculation.

Table 2. Effect of phage application times on percentage of infected grains and rate of filled grains.

Treatments	The percentage of infected grains (%)			AUDPC	Rate of filled grains (%)
	5 dai	10 dai	15 dai		
T1	11.9 ^{c}	15.7^{c}	17.7^{c}	152^{c}	87.8 ^{a}
T2	10.2^{c}	11.3^{c}	11.8^{c}	111^{c}	86.6 ^{a}
T3	26.2 ^{b}	35.1^{b}	40.9^{b}	343^{b}	64.6 ^{b}
Control	40.5^{a}	55.1^{a}	60.8^{a}	529^{a}	41.5 ^{c}

Values in the same column with the same letter are not significant difference (p>0.05). dai: day after inoculation. T1: Spraying 2 hour before inoculation; T2: the combination of spraying 2 hours before pathogen inoculation and 5 days after pathogen inoculation; T3: Spraying 5 days after inoculation.

In short, two treatments (i.e. spraying 2 hours before pathogen inoculation and combined spraying 2 hours before and 5 days after pathogen inoculation) showed potential in reduction of grain rot disease incidence and better yield protection than treatment spraying at 5 days after pathogen inoculations as well as control (Figure 2). Reasonable, application of phage before pathogen infection caused phage contact easier to the host of bacteria and reduced bacterial density before its infection and multiplication inside the plant tissue. Similarly, Civerolo and Keil (1969, as cited in Jones et al., 2012) also reporter that disease reduction of peach bacterial spot was higher if phage treatment was applied one hour or one day before inoculation of the pathogen; and slight disease reduction when phage was applied one hour after inoculation and no effect if applied one day later. In another study, Bergamin and Kimati (1981, as cited in Jones et al., 2012) investigated the effect of timing on the efficacy of phage treatment in greenhouse trials as well as black rot of cabbage (caused by *Xanthomonas campestris* pv. *campestris)* and bacterial spot of pepper (caused by *X. campestris* pv. *vesicatoria)*. The greatest disease reduction occurred when phages were applied on the day of inoculation in both pathosystems. Therefore, the result of this experiment suggested that phages application for controlling bacterial diseases should be applied at critical times as soon as bacteria present on the host and before its infection.

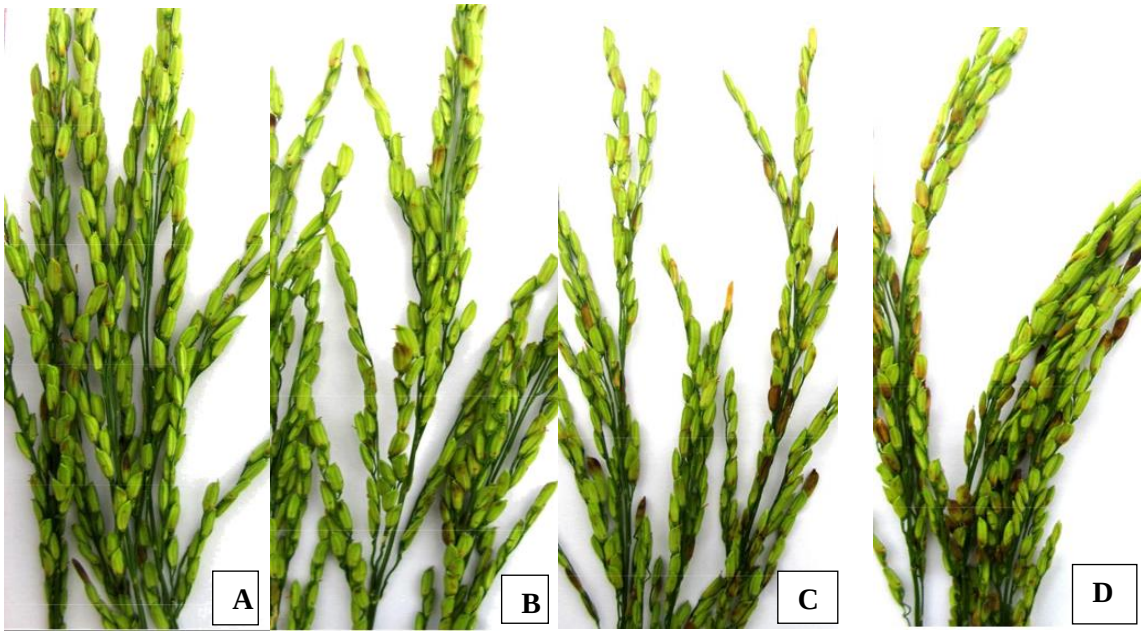

Figure 2. Effect of phage application times in controlling bacterial grain rot of rice in the greenhouse conditions at 5 days after pathogen inoculation

(A): spraying at 2 hours before inoculation; (B): spraying at 2 hours before and 5 days after inoculation; (C): spraying at 5 days after inoculation; (D): control

4. CONCLUSIONS

Applicating of phage ΦBurAG58 of titers 10^5- 10^8 PFU/mL can reduce grain rot disease on rice caused by *Burkholderia glumae*, in which phage titer of 10^8 PFU/mL was the best effective treatment and better protecting rice yield parameter. In addition, two phage applications on flowers as one time at 2 hours before pathogen inoculation or two times application at 2 hours before and 5 days after pathogen inoculation have more disease protection efficacy than phage application at 5 days after pathogen inoculation. The finding suggested that phage suspension at titer 10^8 PFU/mL should be applied on rice flowers in the heading stage once before flowering or twice (including another at 5 days after flowering) to control rice bacterial grain rot disease.

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