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Efficacy of bacteriophages in controlling bacterial vascular wilt caused by *Ralstonia solanacearum* Smith on eggplants

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ABSTRACT

The objective of the study was to select promising bacteriophages for lysis Ralstonia solanacearum in vitro and evaluate their ability to prevent bacterial vascular wilt on eggplants under greenhouse conditions. Primary selection of promising bacteriophages from four bacteriophages, Φ54, Φ60, Φ67, and ΦBT on Ralstonia solanacearum were isolated from eggplant based on plaque diameter and phage multiplication in vitro. The result found that the three bacteriophages (Φ54, Φ67, and ΦBT) expressed plaque diameter over 7.00 mm at 48 hours, and phage titer with log (pfu/ml) reached over 7.00. The efficacy of these phages in controlling bacterial wilt on eggplants under greenhouse conditions through applying each bacteriophage or cocktail of three phages suspension two times (10^{10} pfu/ pot/ each time) at before pathogen inoculation and 7 days after pathogen inoculation through soil drenching was tested. The results showed that all bacteriophages either applied singly or as a mixture of the three phages were effective in the prevention of bacterial wilt disease. In particular, phage Φ BT showed the highest disease reduction and better than bacteriocide treatment applied with Starner 20 WP.

1. INTRODUCTION

Ralstonia solanacearum is an important pathogen that causes bacterial vascular wilt in over 250 plant species worldwide (Landry et al., 2020). It is referred to as the "*Ralstonia solanacearum* species complex" syndrome (Peeters et al., 2013). In the context of climate change such as temperature increase, drought, and water logging (IPCC, 2014, as cited in Aoun et al., 2017), the bacterial genus *Ralstonia* spp. is considered one of the most harmful pathogens, impacting food security worldwide. To prevent this disease, using agrichemical controls is not highly effective because the bacteria attack the xylem vessels, and the chemical method for this disease mainly through soil drenching is harmful to soil microbial communities. Biological control is considered as an eco-friendly method to help reduce toxic chemical usage. Phage therapy has been successfully noted in controlling many bacterial diseases in plants (Jones et al., 2007). Through the limitation of the resistant host bacteria in nature, a phage cocktail is recommended for controlling the disease under field conditions (Balogh et al., 2010; Torres-Barceló, 2018; Laanto et al., 2020). Therefore, this study aimed to select promising bacteriophages to effectively manage bacterial vascular wilt disease on eggplants under greenhouse conditions.

2. MATERIALS AND METHOD

2.1. Evaluation of the lytic ability of bacteriophages on *Ralstonia solanacearum in vitro*

The aim of the experiment was to selected promising phages based on two characteristics: plaque diameter and multiple abilities on host bacterium *R. solanacearum* cause bacterial wilt on eggplants.

Sources of bacteriophages and *Ralstonia solanacearum*: four bacteriophages as Φ54 (isolated on chili), Φ60 (isolated on chili), Φ67 (isolated on daisy), and ΦBT (isolated on eggplant), and two bacterial strains (*R. solanacearum* 1 isolated on eggplant, Rs1); *R. solanacearum* 2 (isolated on bitter melon, Rs2), these microbial sources were supplied by Department of Plant Protection, College of Agriculture, Can Tho University.

Culturing bacteriophage: Each phage i. e., Φ54, Φ60, Φ67, and ΦBT were multiplied on King's B 0.8% agar medium containing host bacterium Rs2 (isolated from bitter melon). After 24 hours, 10 ml sterile distilled water was added for harvesting phage suspension, elimination of host bacterium from the suspension by adding 5% chloroform (v/v) and centrifugation at 6,000 rpm for 5 minutes. Each phage supernatant was transferred into a new tube and determined phage titer by dilution method and plating on King B 0.8 % agar containing Rs2, all phage suspensions were diluted to titer 10^8 pfu/ml used for selection of promising phage on Rs1 (isolated from eggplant) based on plaque size and phage titer.

The experiment followed a completely randomized design with four treatments and three replicates. The experiment was conducted on Petri plates by adding 100 µl bacterial suspension and 100 µl of each bacteriophage suspension at different titer diluted from 10^6 pfu/ ml onto the plates 10 ml King's B medium 0.8% agar containing host bacterium Rs1 at 50°C, the plates were mixed well and then placed in room temperature.

Data collection: Recording plaque size of each phage on Rs1 at 24, 36 and 48 hours (ten plaques/ each replicate) and recording titer (pfu/ ml) of each phage forming on Rs1 after 24 hours.

2.2. Evaluation of efficiency of bacteriophage for controlling bacterial vascular wilt caused by *Ralstonia solanacerum* in eggplants under greenhouse conditions

The aim of the experiment was to determine the treatment of particular phage or phage cocktail in controlling bacterial wilt on eggplants under greenhouse conditions.

The experiment was followed a completely randomized design with six treatments (i. e., three treatments treated with a particular phage (i.e. ΦBT, Φ54, Φ67), phage cocktail (mixture of three phages), bacteriocide treatment (Starner 20WP, Sumitomo Chemical company) and a control treatment). Each treatment included 4 replicates.

Plant preparation: Eggplant seeds (variety TN 106, Trang Nong seed company) were sown in plastic pots containing 2 kg of sterilized soil at d density of 10 well-growing seedlings per pot.

Preparation of *R. solanacearum*: Rs1 strain was cultured on petri dishes containing King's B 2% agar medium for 48 hours, harvest bacterial suspension, then diluted the bacterial suspension to OD_{600nm} = 0.3 (corresponding to a density of 8 x 10^8 cfu/ml).

Preparation of bacteriophage: Each bacteriophage was cultured into King'sB 0.8% agar medium for 48 hours, treated 5% chloroform (v/v), and centrifuged at 6,000 rpm for 5 min. Phage titer (pfu/ ml) of each phage suspension was determined, then diluted to the density at 10^8 pfu/ ml for each bacteriophage. A phage cocktail was prepared by mixing the three phages (10^8 pfu/ ml) with each phage density being one-third in the suspension.

Pathogen inoculation, phage, and bacteriocide treatment: At 30 days after sowing when the plants had 5 to 6 leaves, all treatments were inoculated with 100 ml/pot of Rs1 suspension (OD_{600nm} = 0.3,corresponding to 8 x 10^8 cfu/ml). For the phage treatment, phage suspension was applied by soil drenching two times i.e. one hour before pathogen inoculation and 7 days after pathogen inoculation with 100 ml per pot. For bacteriocide treatment, Starner 20 WP was applied 100 ml/ pot at the recommended dose when the first symptom occurred.

Data recording: Percentage of infection (%) and disease scale as described by Ateka et al., (2001), included 6 scales (0 = no symptoms, 1 = the first lower leaf wilt, 2 = the 2nd or 3rd leaves wilt, 3 = all the leaves wilt except the top leaves, 4 = all leaves wilt, 5 = plant dead) were recorded at different time intervals after pathogen inoculation. In addition, calculating disease progress through Area Under the Disease Progress Curve (AUDPC) (Shanner & Finney, 1977).

Data statistics: The data were processed by Microsoft Excel and statistically analyzed using MSTATC software. Mean of treatment was ranked using Duncan Multiple range test.

3. RESULTS AND DISCUSSION

3.1. The lytic ability of bacteriophage on *Ralstonia solanacearum* Rs1 *in vitro*

Plaque size of four the bacteriophage formations on Rs 1 lawn on King B 0.8% agar is shown in Table 1. The result showed that all four bacteriophages have the ability to lyse the Rs1 with different levels under room conditions. In which, three bacteriophages as ΦBT, Φ54, and Φ67 gave a higher plaque diameter in the range from 6.32 to 6.95 mm at 36 hours, and 7.12 to 7.50 mm at 48 hours, which were significantly higher different from that of Φ60, with smaller plaque size 4.41 and 4.54 mm, respectively (Table 1, Fig. 1).

Table 1. The lytic ability of some bacteriophages on Ralstonia solanacearum 1 invitro

Treatment	Plaque diameter (mm)		Log (pfu/ml)
	36 hours	48 hours	24 hours
ΦBT	6.95 [a]	7.37 [a]	7.00 [b]
Φ54	6.77 [a]	7.12 [a]	7.17 [b]
Φ60	4.41 [c]	4.54 [b]	6.94 [b]
Φ67	6.32 [b]	7.50 [a]	7.88 [a]

Mean values in the same column followed with the same letter are not significantly different (p<0.05).

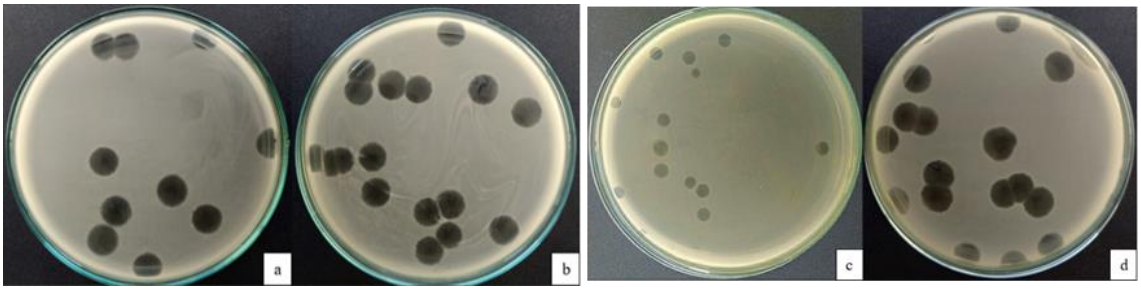

Figure 1. Plaque diameter of four bacteriophages on R. solanacearum 1 lawn after 36 hours (a) ΦBT, (b) Φ54, (c) Φ60, (d) Φ67

In addition, the phage titer obtained when culturing separately four bacteriophages on Rs 2 was 10^6 pfu/ml, but when plating on lawn Rs 1 these phage titer formations expressed differently and with higher titer when plating on Rs2, which indicated the Rs 1 was more sensitive to the four phages than to Rs 2. In addition, Φ67 reached log phage titer 7.40 (pfu/ml) which was significantly higher than the remaining three bacteriophages (i.e. ΦBT, Φ54, Φ60) with log (pfu/ml) 7.00; 7.17; and 6.94, respectively. Therefore, based on plaque diameter and high phage titer formation on Rs1, three bacteriophages as ΦBT, Φ54, and Φ67 were selected as promising phages for further investigation of their disease control efficacy of bacterial vascular wilt on eggplants in greenhouse conditions.

3.2. The bacteriophage efficiency for controlling bacterial vascular wilt caused by *Ralstonia* solanacearum in eggplants

Bacterial wilt symptoms on eggplants occurred after 4 days following soil drenching with Rs1. Four phage treatments and bacteriocide treatments showed a significantly lower percentage of infection and disease than the untreated control through over allobservation intervals (Table 2).

On the percentage of infection from 4 - 12 dai, all treatments showed disease reduction with significantly lower percentage of infection than the control treatment. Especially at 12 days after inoculation, the percentage of infection in the control treatment reach 85% while that of phage ΦBT and Φ54 treatments showed only 15.00 % and 17.50 %, respectively, and significantly lower than those of phage Φ67 and bacteriocide treatment

(Starner 20 WP) with 42.50%, next was the phage cocktail treatment with a percentage of infection of 30%.

Similarly, all treatments expressed a lower disease scale than that of the negative control. Especially at 12 dai, phage ΦBT, Φ54, and the phage cocktail showed better disease reduction, with disease scales ranges from about 0.28 - 0.88, which was significantly lower than bactericide and the control treatment with disease scales of 1.63 and 3.00, respectively.

Table 2. The ability of bacteriophage to prevent bacterial vascular wilt on eggplants under greenhouse conditions

Treatments	Percentage of infection (%)			Disease scale		
	4 dai	8 dai	12 dai	4 dai	8 dai	12 dai
ΦBT	0.0^{b}	0.00^{d}	15.0^{c}	0.0 0^{b}	0.00^{d}	0.28^{d}
Φ54	5.0^{b}	12.5^{c}	17.5^{c}	0.05^{b}	0.18^{cd}	0.55^{cd}
Φ67	2.5^{b}	20.0^{bc}	42.5^{b}	0.03^{b}	0.23^{cd}	1.10^{bc}
Phage cocktail	2.5^{b}	15.0^{bc}	30.0^{bc}	0.03^{b}	0.28^{c}	0.88^{cd}
Oxolinic acid	5.0^{b}	30.0^{ab}	42.5^{b}	0.05^{b}	0.58^{b}	1.63^{b}
Control	27.5 ^{a}	47.5^{a}	85.0^{a}	0.28^{a}	1.35^{a}	3.00^{a}

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

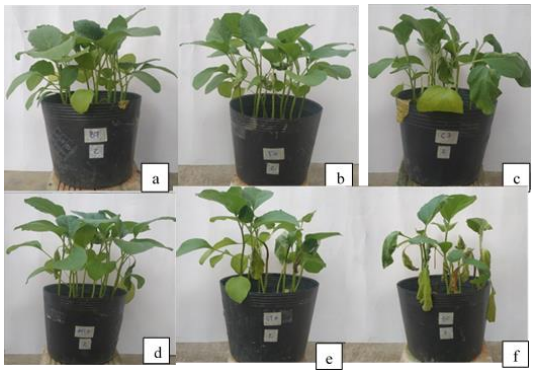

Figure 2. The efficacy of bacteriophages in controlling bacterial vascular wilt on eggplant caused by *Ralstonia solanacearum* at 10 days after inoculation under greenhouse conditions (a) ΦBT, (b) Φ54, (c) Φ67, (d) phage cocktail, (e) Oxolinic acid, (f) control

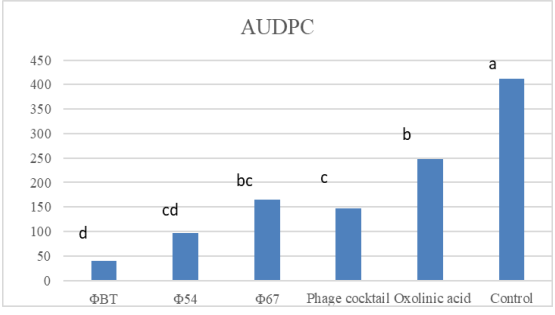

Figure 3. Bacterial wilt disease progress (AUDPC) caused by *Ralstonia solanacearum* on eggplants of treatments under greenhouse conditions

In addition, on AUDPC of all four phage treatments and Starner were significantly lower than the untreated control (Figure 3), in which ΦBT and Φ54 treatments had the lowest AUDPC correlation and highest disease reduction. In addition, the treatment of Φ54, Φ67, and the phage cocktail showed a similar AUDPC level and was equal to the Oxolinic acid (Figure 3).

Our results show that all three promising phages expressed good efficacy in controlling Ralstonia wilt on eggplants under greenhouse conditions. Phage ΦBT showed the best disease reduction. The result contributes to the knowledge that bacteriophage is a promising agent in controlling plant bacterial diseases, and should be considered as an alternative biological control for prevention of antibiotic resistant bacteria, contributing to the protection of biodiversity (Fujiwara, 2011; Jassim & Limoges, 2014). The lytic phage can be an effective antibacterial agent due to its specificity against a particular bacterial species and lack of impact on other microflora. Therefore, we can use phage in combination with the use of antagonistic bacteria that increase the pressure on pathogens without harming beneficial bacteria (Jones et al., 2007, Köhl et al., 2019). The more plaque formation that is involved in recognition, adsorption, and their interaction during attachment to the host cell wall; Thus, different host bacterium could lead to different levels of plaque formation. According to Nobrega et al. (2018), phages will infect variability on different host bacterium which is determined by the specific structures that they use to target

bacterial cells. For example, tailed phages use a broad range of receptor-binding proteins, such as tail fibers, tail spikes, and the central tail spike, to target their cognate bacterial cell surface receptors. Therefore, the plaque diameter and titer on *Ralstonia solanacearum* causing disease in eggplants are different levels. Regarding the ability of phage to prevent bacterial vascular wilt on eggplants under greenhouse conditions expressed a very promising method for controlling bacterial wilt disease which is a devasting pathogen of many crops. Applying single phage ΦBT or phage cocktail (ΦBT, Φ54, and Φ67) suspension (10^{10} pfu/ pot) two times before and after *Ralstonia solanacearum* infection by soil drenching will provide better disease control than bacteriocide treatment. The same result recorded by Fujiwara et al. (2011), investigated soaking combined with soil drenching one month after sowing with phage φRSL1 suspension (1.3×10^{10} PFU/pot) and showed effectiveness in controlling bacterial wilt on tomatoes caused by *R. solanacearum*. Therefore, our results indicated that phage application to soils can prevent bacterial wilt disease in eggplants caused by *Ralstonia solanacearum*. This method reduce antibiotics or chemical usages in agriculture which can negatively impact the environment and human health.

4. CONCLUSIONS

Study on the selection of promising bacteriophage against bacterial vascular wilt caused by *Ralstonia solanacearum* on eggplant, three promising phages (ΦBT, Φ54, Φ67) were selected based on big plaque size in vitro condition. These application of single phage or a cocktail of the three phages also express good efficacy in preventing bacterial wilt symptoms in eggplants greenhouse conditions by soil drenching. In which, phage ΦBT isolated from eggplants show the highest ability to reduce disease better than bacteriocide treatment.

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