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

Keywords

Bacteriophage, eggplant, phage cocktail, *Ralstonia solanacearum*

1. INTRODUCTION

*Ralstonia solanacearum* is one of the important pathogens that cause bacterial vascular wilt in over 250 plant species worldwide (Landry *et al*., 2020), which 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 harmful pathogens that are warned because of their impact on food security in the world. To prevent this disease, using agrichemical control is not highly effective because the bacteria have attacked the xylem vessels and the chemical method for this disease is mainly through soil drenching which is harmful and affects the soil microbial community. Biological control is considered as an eco-friendly method to help reduction of toxic chemical usage in agricultural environment. Phage therapy had been recorded successfully in controlling main ly 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 in field conditions (Balogh *et al*., 2010, Torres-Barceló, 2018, Laanto *et al*., 2020). Therefore, the study aimed to select promising bacteriophages to effectively manage bacterial vascular wilt disease on eggplants in greenhouse conditions.
2. MATERIALS AND METHOD
2.1. Evaluation of the lytic ability of bacteriophages on Ralstonia solanacearum in *in vitro*

The aim of the experiment is a selection of 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 was 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 10^6 pfu/ml used for selection of promising phage on Rs1 (isolated from eggplant) based on plaque size and phage titer.

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

The aim of the experiment is to determine the treatment of particular phage or phage cocktail in controlling bacterial wilt on eggplants in 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 Control treatment). Each treatment included 4 replicates.

**Plant preparation:** eggplant seeds (variety TN 106, Trang Nong seed company) were sown in a plastic pot containing 2 kg sterilized soil and kept 10 well-growing seedlings per pot.

**Preparation of R. solanacearum:** Rs1 strain was cultured on petri dish 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’s B 0.8% agar medium for 48 hours, treated with 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 of 10^8 pfu/ml for each bacteriophage. A phage cocktail was prepared by mixing 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 plant has the fifth to sixth 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 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 occurs.

**Data recording:** percentage of infection (%) and disease scale as described by Ateka et al., (2001), including 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 courses 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 by 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 *in vitro*

Plaque size of four bacteriophage formations on Rs 1 lawn on King B 0.8% agar was shown in Table 1, the result showed that all four bacteriophages can 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 in *in vitro*

| 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 by 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 is more sensitive to four phages than Rs 2. In addition, Φ67 reach log phage titer 7.40 (pfu/ml) 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 occur at 4 days after soil drenching with Rs1. Four phage treatments and bactericide treatment showed a significantly lower percentage of infection and disease scale than untreated control through all-time course observation (Table 2).

On the percentage of infection from 4 - 12 dai, all treatments showed disease reduction with a 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 had only 15.00 % and 17.50 %, respectively, and significantly lower than those of phage Φ67 and bactericide treatment (Starner 20 WP) with 42.50%, next was phage...
cocktail treatment with a percentage of infection was 30%.

Similarly, all treatments expressed a lower disease scale than that of the negative control. Especially at 12 dai, phage ΦBT, Φ54, and phage cocktail showed better disease reduction, with disease scales ranged about 0.28 - 0.88, which were 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 in the greenhouse conditions

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

Values in the same column followed by 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 in the 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 phage cocktail had a similar AUDPC level and was equal to the Oxolinic acid (Figure 3).

So in this study, all three promising phages expressed good efficacy in controlling Ralstonia wilt on eggplants in greenhouse, and phage ΦBT showed the best in disease reduction. The result contributes in the fact that bacteriophage is a promising agent in controlling plant bacterial diseases, and should be considered as an alternative biological control for the prevention of antibiotic-resistant bacteria, contributing to the protection of biodiversity in the ecosystem (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 extremely increase the pressure on pathogens without harming the beneficial bacteria (Jones et al., 2007, Köhl et al., 2019). Therefore, 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 eggplant are different levels. Regarding the ability of phage to prevent bacterial vascular wilt on eggplants in greenhouse conditions expressed a very promising method for controlling bacterial wilt disease which is a devasting pathogen of almost 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 give better disease control than bacteriocide treatment. The same result recorded by Fujiwara et al. (2011), seek soaking combined with soil drenching one month after sowing with phage φRSL1 suspension (1.3 \times 10^{10} PFU/pot) shows effectiveness in controlling bacterial wilt on tomato caused by R. solanacearum. Therefore, this result indicated that phage application into the soil can excellently prevent bacterial wilt disease on eggplant caused by Ralstonia solanacearum, this is a friendly method to help reduce antibiotics or chemical usage in agriculture which negatively impacts 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 conditions. The application of single phage or cocktail of three phages also expresses good efficacy in preventing bacterial wilt symptoms on eggplant in 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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