BACTERIOPHAGES IN PREVENTING BACTERIAL WILT ON MARIGOLD CAUSED BY *Ralstonia solanacearum*

Nguyen Thi Thuy Duong, Le Vo Quynh Nhu, Nguyen Van Linh, Nguyen Van Duong, Nguyen Thi Thu Nga, Doan Thi Kieu Tien*

Department of Plant Protection, College of Agriculture, Can Tho University

*Corresponding author: dtktien@ctu.edu.vn

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ABSTRACT

Bacterial wilt caused by *Ralstonia solanacearum* is a severe disease in many vegetable and ornamental plants. This research was conducted to screen promising bacteriophages for controlling bacterial wilt in marigold under laboratory and greenhouse conditions. The lytic capability of twenty bacteriophages parasited nine strains of *R. solanacearum* causing bacterial wilt in marigolds and other crops. The results showed that all bacteriophages were able to parasitize all bacterial strains examined. A lytic ability assessment of the twenty bacteriophages lysis on *R. solanacearum* on marigolds revealed that three phages ΦOM, Φ1G, and ΦĐT3 gave strong bacterial lysis through high titer multiplication. Additionally, phage Φ2D and ΦOM observed large plaque diameter compared to others. The three phages ΦOM, Φ1G, and ΦĐT3 were selected for further evaluation of their effectiveness in controlling bacterial wilt caused by *R. solanacearum* in marigold under greenhouse conditions. In which, phage ΦOM provided the most effective disease control and showed a significantly lower disease incidence compared to other treatments.

***Keywords**:* Bacteriophage, Marigold, *Ralstonia solanacearum*, *Tagetes erecta*

THỰC KHUẨN THỂ PHÒNG BỆNH HÉO XANH TRÊN CÂY VẠN THỌ DO VI KHUẨN *Ralstonia solanacearum*

Nguyễn Thị Thùy Dương, Lê Võ Quỳnh Như, Nguyễn Văn Linh, Nguyễn Văn Dương, Nguyễn Thị Thu Nga, Đoàn Thị Kiều Tiên*

Khoa Bảo vệ thực vật, Trường Nông nghiệp, Đại học Cần Thơ

*Tác giả liên hệ: dtktien@ctu.edu.vn

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TÓM TẮT

Bệnh héo xanh do vi khuẩn *Ralstonia solanacearum* là nguyên nhân gây bệnh trên nhiều loại rau và cây hoa kiểng. Nghiên cứu đã được tiến hành để sàng lọc các thể thực khuẩn có triển vọng nhằm kiểm soát bệnh này trên cây vạn thọ trong điều kiện phòng thí nghiệm và nhà lưới. Khả năng kí sinh của hai mươi dòng thể thực khuẩn trên chín chủng *R. solanacearum* gây bệnh héo xanh ở vạn thọ và các loại cây trồng khác. Kết quả cho thấy tất cả các thể thực khuẩn đều có khả năng ký sinh trên tất cả các chủng vi khuẩn được kiểm tra. Đánh giá khả năng phân giải của hai mươi dòng thể thực khuẩn trên *R. solanacearum* trên vạn thọ cho thấy ba thể thực khuẩn ΦOM, Φ1G và ΦĐT3 cho khả năng phân giải vi khuẩn mạnh thông qua mật số cao. Ngoài ra, thể thực khuẩn Φ2D và ΦOM quan sát thấy đường kính đốm tan lớn so với các thể khác. Ba thể thực khuẩn ΦOM, Φ1G và ΦĐT3 đã được lựa chọn để đánh giá thêm hiệu quả phòng trừ bệnh héo xanh vi khuẩn do vi khuẩn *R. solanacearum* gây ra trên cây cây vạn thọ trong điều kiện nhà lưới. Trong đó, thể thực khuẩn ΦOM cho hiệu quả phòng trừ bệnh tốt nhất và tỷ lệ mắc bệnh thấp hơn đáng kể so với các nghiệm thức xử lý khác.

***Từ khóa**:* Cây vạn thọ, Thực khuẩn thể, *Ralstonia solanacearum*, *Tagetes erecta*

1. INTRODUCTION

Bacterial wilt caused by *Ralstonia solanacearum* Smith is one of the most plant pathogens in agriculture that causes significant destructive and hard properties to control affecting plants (Nguyen Thi Thu Cuc and Tran Thi Thu Thuy, 2014). *R. solanacearum* is a Gram - negative bacterium known to cause wilt in more than 310 plant species across 53 different families (Wang et al., 2023). Currently, the use of bacteriophages for bacterial disease management is attracting increasing attention as a promising component of integrated disease management (IDM) strategies. Bacteriophages offer high specificity and effectiveness in targeting and inactivating plant pathogenic bacteria (Balogh, 2002; Ye et al., 2019). Due to their host - specific parasitism and environmentally friendly mode of action, phages are considered an effective biological control method, especially in the context of increasing resistance of plant pathogens to antibiotics and chemical pesticides (Umrao & Kaistha, 2021). In fact, bacteriophages have been successfully applied to manage bacterial wilt on crops such as ginger and potato, with a substantial number of studies focusing on Solanaceous crops (Biosca et al., 2021; Alvarez et al., 2022; Hasanien et al., 2024). In 2019, Alvarez et al. demonstrated that the application of bacteriophages (ΦRsoP–WF2, ΦRsoP–WM2, and ΦRsoP–WR2) significantly reduced the population of *R. solanacearum* causing wilt on tomato (Álvarez et al., 2019). Similarly, Ramirez et al. (2020) reported that a phage cocktail consisting of strains M5 and M8 effectively controlled Moko disease in banana caused by *R. solanacearum* (Ramírez et al., 2020). In Vietnam, Nguyen Thi Thuy Hang et al. (2021) applied bacteriophages to drenching soil, demonstrating effective control of bacterial wilt on marigold (*Tagetes erecta*). More recently, Tri et al. (2025) also confirmed the efficacy of bacteriophages in managing bacterial wilt across tomato. In this study, development of effective phage - based management strategies for bacterial wilt on marigold is essential to evaluate the parasitic potential and propagation ability of candidate bacteriophage.

2. MATERIALS AND METHODS

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

Materials: The experiment utilized nine *R. solacearum* bacterial strains and twenty phages, provided by the Department of Plant Protection, College of Agriculture, Can Tho University.

Testing the host range of phages: Each phage was individually propagated on 0.8% King's B medium containing its corresponding host bacterium for 24 hours. The phage suspensions were harvested by treating the cultures with 5% chloroform, followed by centrifugation at 6000 rpm for 5 minutes. The resulting supernatants, containing purified phages, were collected. A 10 µL aliquot of each phage suspension was spotted onto prelabeled sectors of Petri dishes containing 10 mL of King's B soft agar medium (0.8% agar), which had been amended with 100 µL of *R. solanacearum* suspension (OD_{600nm} = 0.3) previously prepared. The plates were dried, kept in the dark, and incubated at room temperature for 24 hours. This procedure was repeated identically for all nine bacterial strains.

Data collection: The number of *R. solanacearum* strain lysed by each phage

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and the number of phages capable of lysing each bacterial strain were recorded.

2.2. Evaluation of the lytic activity of bacteriophages against *Ralstonia solanacearum*

The experiment was designed in a completely randomized design (CRD) with one factor, consisting of twenty phages with three replications per treatment. The phage suspensions were diluted to three levels: 10^{-6}, 10^{-7}, and 10^{-8}. Added 100 µL of bacterial suspension (OD_{600nm} = 0.3) and 100 µL of each diluted phage suspension was added to 10 mL of soft agar King's B medium (0.8%). The plates were incubated at room temperature in the dark for 24 hours, after which plaque formation was observed.

The phage titer (PFU/mL) was determined 24 hours after incubation by counting the number of clear plaques and calculating based on the dilution factor. In addition, plaque diameters were measured to assess the lytic capability of each phage. The plaque diameter was recorded by marking 10 randomly selected plaques per plate and averaging their size at 24, 36, and 48 hours after incubation.

Phage titer data were log-transformed using Microsoft Excel Version 13. Statistical analysis was performed using SPSS software Version 29, and treatment means were compared using Duncan's multiple range test.

2.3. Evaluation of effectiveness phages in preventing bacterial wilt on marigold caused by *Ralstonia solanacearum* in the greenhouse conditions

Material: three potential phages were screened from the experiment 2.1 and 2.2

The experiment was arranged in a completely randomized design (CRD) with one factor consisting of six treatments: three individual promising phages, a phage mixture (cocktail of three phages), the chemical bactericide Starner, and an untreated control. Each treatment was replicated three times. Each replicate consisted of one pot containing ten marigold plants, with three kg of soil per pot.

The pathogen inoculation was performed by applying 50 mL of a *R. solanacearum* bacterial suspension (5×10^7 CFU/mL) to each pot at 14 days after sowing. Two hours prior to inoculation, biological control treatments were applied by drenching soil each pot with 50mL of the corresponding phage treatment at a density of 10^8 PFU/mL.

Percentage of infection (%) and Area Under the Disease Progress Curve (AUDPC) (Shanner & Finney, 1977). Statistical analysis was conducted using SPSS software, and mean of treatment was ranked using Duncan Multiple range test.

3. RESULTS AND DISCUSSION

3.1. The results of lytic ability of phages on *Ralstonia solanacearum* strains

A study was conducted to evaluate the parasitic capacity of twenty phages infected nine *R. solanacearum* strains collected from different host plants, including marigold, tomato, basil, sweet basil, and chili. The results presented that all twenty phages were capable of parasitizing 100% of the tested *R. solanacearum* strains associated with bacterial wilt disease. Furthermore, all bacterial isolates obtained high sensitivity to these phages. Actually, Yamada et al., (2007) was isolated four phages from *R. solanacearum*, a soil-borne disease, Gram - negative bacterium that is the causative agent of bacterial wilt in many important crops. Among them, phiRSA1 was able to infect all fifty *R. solanacearum* strains of different races or different biovars tested in this study. Similarly, Ozawa *et al*. (2001) reported that phages isolated from various regions in Japan were also capable of a large parasitizing from different *R. solanacearum* strains. In short, this experiment was performed the role of phages of *R.*

solanacearum can infect a wide different strains of *R. solanacearum* which were isolated from a different host plant.

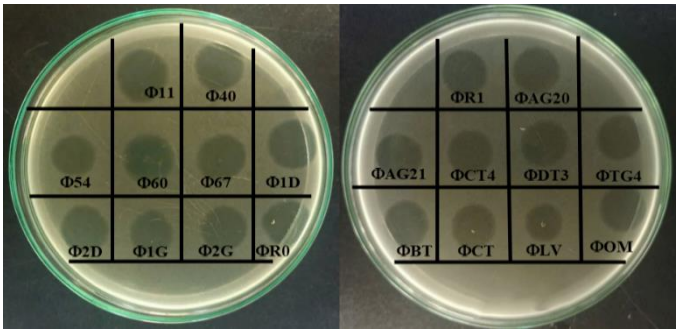

Figure 1. The lytic ability of bacteriophages on *Ralstonia solanacearum* causing bacterial wilt on marigold at 24 hours

3.2. Multiplication and lytic ability of bacteriophages on *Ralstonia solanacearum* causative agent bacterial wilt on marigold

The log of phage titer was presented in the Table 1; it was shown that the ability of twenty phages to replicate on the *R. solanacearum* bacterial strain after 24 hours of incubation differed significantly among the phage isolates at the 1% significance level.

The phage titer (Log PFU/mL) of the twenty phages were replicated on *R. solanacearum* ranged from 8.49 to 9.80. Among them, phage ΦOM gave higher the titer and significant difference from the remaining phages. Following this, phage Φ1G did not differ significantly from ΦĐT3, but showed statistically significant differences compared to others.

The plaque diameter of phages was shown in Table 1; the plaque size of difference phages was observed at several time courses. At 24 hours after inoculation, nine phages (Φ2G, ΦTG4, ΦR1, Φ1G, ΦCT, ΦOM, Φ2D, ΦĐT3, and Φ11) expressed significantly larger clear zone diameters, ranging from 3.13 mm to 3.40 mm, compared to the remaining treatments. Similarly, at 36 hours after inoculation, plaque diameters of all twenty phages increased markedly compared to the 24 - hour time point. Among them, strains Φ2D and ΦOM continued to lysis bacterial lawn layer, comparable to phage Φ2G, ΦTG4, ΦR1, Φ1G, ΦCT, ΦĐT3, and Φ11, and significantly different from the remaining others. By 48 hours after inoculation, the lysis zone diameters of the phages continued to increase. Notably, ΦOM maintained the highest plaque diameter at 3.72 mm, though the difference was not statistically significant compared to others at the 1% significance level.

To sum up, the three recorded time points, all twenty phages demonstrated the ability to lyse *R. solanacearum*, the causal agent of bacterial wilt in marigold. Among them, phages ΦOM, Φ2D, Φ2G, ΦTG4, ΦCT, ΦĐT3, and Φ11 gave significantly greater lytic activity compared to that of other strains. This statistical difference may be attributed to variation in the lysis timing among phages, leading to differences in the number of virions released and the size of the resulting lysis zones. These findings were published with the study by Gallet et al. (2011), which reported that virion morphology can influence the diameter of lysis zones phages that release

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virions more rapidly tend to produce larger lysis zones.

Table 1. The lytic and replication ability of twenty bacteriophages when culture on *Ralstonia solanacearum*

Treatments	Plaque size (mm)			Log phage titer(pfu/mL)
	24 HAI	36 HAI	48 HAI	
Φ2G	3.13^{a}	3.32^{ab}	3.37^{abc}	9.11^{c-f}
ΦTG4	3.15^{a}	3.37^{ab}	3.37^{abc}	8.50^{h}
ΦR1	3.28^{a}	3.32^{ab}	3.33^{bc}	9.17^{cde}
Φ1G	3.20^{a}	3.33^{ab}	3.33^{bc}	9.54^{b}
Φ54	3.10^{ab}	3.22^{b}	3.27^{c}	8.89^{fg}
ΦCT	3.30^{a}	3.45^{ab}	3.47^{abc}	9.26^{cd}
ΦOM	3.37^{a}	3.60^{a}	3.72^{a}	9.80^{a}
Φ2D	3.40^{a}	3.63^{a}	3.68^{ab}	8.49^{h}
ΦĐT3	3.35^{a}	3.42^{ab}	3.45^{abc}	9.35^{bc}
Φ11	3.23^{a}	3.37^{ab}	3.38^{abc}	9.22^{cd}
ΦLV	2.22^{def}	2.32^{efg}	2.40^{e-h}	9.24^{cd}
ΦBT	2.02^{fg}	2.17^{fg}	2.30^{fgh}	8.94^{efg}
ΦR0	2.30^{def}	2.43^{def}	2.50^{efg}	9.10^{c-f}
Φ67	1.87^{g}	2.02^{g}	2.07^{h}	9.05^{def}
Φ21	2.13^{efg}	2.17^{fg}	2.25^{gh}	8.75^{f}
ΦCT4	2.38^{de}	2.60^{cde}	2.62^{def}	9.05^{def}
Φ20R	2.48^{cde}	2.62^{cde}	2.70^{de}	9.20^{cde}
Φ60	2.5^{cd}	2.72^{cd}	2.72^{de}	9.26^{cd}
Φ40	2.80^{bc}	2.90^{c}	2.90^{d}	9.25^{cd}
Φ1D	2.18^{efg}	2.30^{efg}	2.32^{fgh}	9.17^{cde}
P value	0.00	0.00	0.00	0.00

$^{a, b:}$ Mean followed by a different letter in the same column to differ significantly (Duncan's test; p- value (0.01) HAI: hour after inoculation

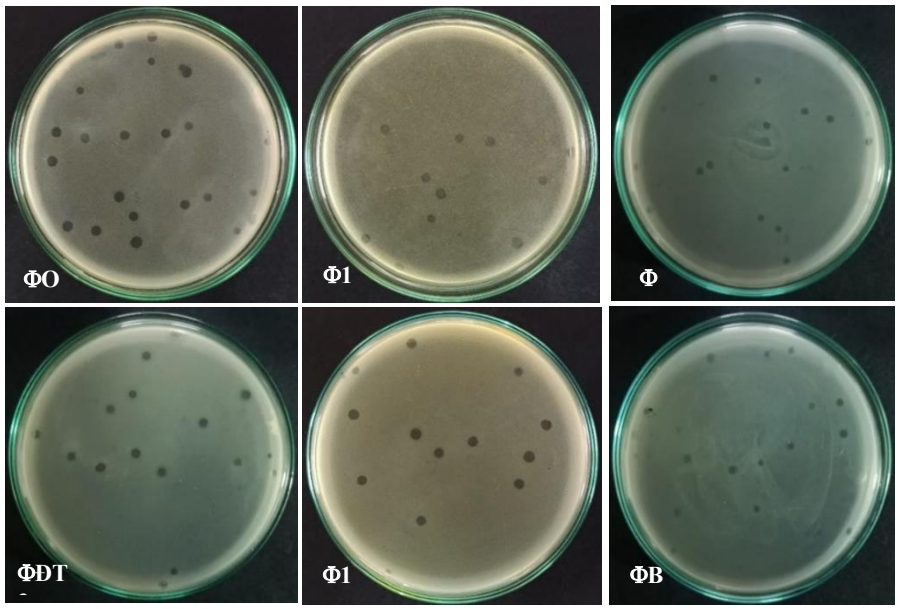

Figure 2. Different phages replication and plaque size of phages formation when culture on *Ralstonia solanacearum* at 10^{-7} dilution at 24 hours of incubation

3.3. Effectiveness phages in preventing bacterial wilt on marigold caused by *Ralstonia solanacearum* under the greenhouse conditions

The effectiveness of bacteriophage treatment in controlling bacterial wilt on marigold plants was evaluated based on disease incidence at 7 and 10 days after inoculation. At 7 days after inoculation (DAI), initial symptoms of bacterial wilt were observed in all treatments. Among them, the phage ΦOM treatment showed the lowest disease incidence, with a statistically significant difference compared to the other treatments. At 10 DAI, disease incidence increased across all treatments compared to day 7. However, the ΦOM treatment continued to exhibit the lowest disease incidence, which was significantly different from the remaining treatments.

Regarding the AUDPC, which reflects the cumulative disease incidence over time, the ΦOM treatment recorded the lowest AUDPC value, with statistically significant differences compared to the other treatments.

Table 3. Bacterial wilt incidence and AUDPC of treatment on Marigold in different time points

Treatment	Disease incidence (%)		AUDPC
	7 DAI	10 DAI	
Φ1G	16.67^{abc}	66.67^{a}	125.00^{a}
ΦOM	6.67^{c}	40.00^{b}	70.00^{b}
ΦĐT3	10.00^{bc}	70.00^{a}	120.00^{a}
Cocktail phage	26.67^{a}	66.67^{a}	140.00^{a}
Starner	13.33^{bc}	56.67^{b}	105.00 ^{ab}
Control	20.00^{ab}	63.33^{a}	125.00^{a}
P value	0.028	0.013	0.026

$^{a, b:}$ *Different letters in each line indicate different means significantly at α = 0.05. DAI: day after inoculation*

Figure 4. The level of bacterial wilt infection in the treatments on marigold in greenhouse at 10 days after inoculation

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Based on the disease incidence results, soil drenching with phage ΦOM suspension at titer 10^8 pfu/mL gave effective control of bacterial wilt caused by *R. solanacearum* in marigold plants. Similarly, these findings have been reported in various studies on the use of bacteriophages for managing bacterial wilt in different crops both globally and in Vietnam (Ozawa et al., 2001; Balogh, 2002; Huynh Huu Tri et al., 2025). These results also confirmed the role of bacteriophages in controlling bacterial wilt caused by *R. solanacearum* in marigold plants.

4. CONCLUSION

Three bacteriophages of ΦOM, Φ1G, and ΦĐT3 expressed the ability to lyse *R. solanacearum*, the causal agent of bacterial wilt in marigold through high replication titers and clear plaque diameters. Among them, the phage ΦOM expressed a higher efficacy in reducing bacterial wilt incidence in marigold under greenhouse conditions. The study indicated that phage therapy can play an important role in controlling bacterial wilt on marigold.

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