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Isolation and characterization of two novel phages, ΦRspv1 and ΦRspv2, with a potential for biocontrol of *Ralstonia solanacearum*

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Abstract

Bacteriophages offer an effective biocontrol strategy, presenting an environmentally friendly alternative to combat the issues of antibiotic and copper-based bactericide resistance in bacterial phytopathogens. In this study, two phages targeting *Ralstonia solanacearun* (designated as ΦRspv1 and ΦRspv2) were identified as potential biocontrol agents against solanaceous wilt, a highly destructive disease causing significant economic losses globally. Both phages demonstrated resistance to chloroform, while ΦRspv1 exhibited a broader host range compared to ΦRspv2 across five isolates of *R. solanaceraum*. The replication cycle for ΦRspv2 was determined to be 90 min. for a single growth cycle, with a burst size of 85 PFU per cell. Morphologically, ΦRspv1 featured icosahedral heads with long tails, characteristic of the *Myoviridae* family, whereas ΦRspv2 displayed a filamentous particle shape typical of *Inoviridae* viruses. Additionally, a Random Amplified Polymorphic DNA (RAPD) analysis using four random primers revealed a diverse nature of the two phages, suggesting their potential utility in disease management strategies. This research unveils two novel virulent phages, and their characterization highlights promising for phage therapy with future investigations aim to field level evaluation against *R. solanacearum.*

Key words: *Bacteriophages, Ralstonia solanacearum,* phage therapy, biocontrol, solanaceous crops

Introduction

Ralstonia solanacearum, the soil-borne bacterium responsible for bacterial wilt, ranks the world's second most damaging phytopathogen (Mansfield *et al*., 2012). *Ralstonia* spp. are Gram-negative, oxidase, and catalase-positive aerobic bacilli thriving in soil and water (Lampropoulos *et al*., 2021). This plant disease wreaks havoc on several agriculturally vital crops like eggplant, tomato, pepper, potato, and ginger, significantly impacting both crop quality and quantity on a global scale (Cai *et al*., 2021). Due to its destructive potential, it is one of the most extensively researched plant pathogens. While the pathogen's virulence has been noted in cooler temperatures, it predominantly thrives in humid and hot climates. During peak infection, *Ralstonia* populations can soar to 10^8 colony-forming units per gram of soil and plant tissue. The bacterium can remain viable for years in water or soil (Alvarez *et al*., 2010). *Ralstonia* typically colonizes the root xylem tissues, infecting the roots of both susceptible and resistant plants through small wounds before swiftly moving to stem tissues. Exopolysaccharides produced by *Ralstonia* cause blockages in the xylem, leading to plant wilting symptoms (Ingel *et al*., 2021). Bacteria spread from plant roots to soil as plants wilt. It triggers a range of virulence factors in different infection states to promote the disease (de Pedro-Jové *et al*., 2021).

Against bacterial wilt, no single management method appears entirely advisable. Despite their use, crop protection chemicals often fall short in providing sufficient control and may pose environmental and human health risks, promote resistance emergence, and incur high costs. Physical treatments prove relatively ineffective, and the impracticality of crop rotations further complicates the management approach. Compounding the challenge, the pathogen exhibits high aggressiveness and endurance in adverse environmental conditions (Álvarez *et al*., 2010; Cao *et al*., 2018).

In addition, extensive use of bactericides and antibiotics has resulted in environmental pollution, the emergence of copper-resistant pathogens, and adverse effects on human health through the food chain (Adrees *et al*., 2015; Costa *et al*., 2018; Lamichhane *et al*., 2018; Kering *et al*., 2019). Copper resistance in plant diseases caused by organisms such as *Pseudomonas* and *Xanthomonas* spp. has complicated pathogen control (Abbasi *et al*., 2015; Colombi *et al*., 2017; Richard *et al*., 2017; Kering *et al*., 2019). Moreover, widespread antibiotic use in agriculture has led to resistance in pathogens like *Erwinia*, *Pseudomonas*, and *Xanthomonas* spp., with instances of streptomycin resistance reported (Kering *et al*., 2019).

Various alternative treatments have been explored for bacterial wilt control, encompassing the utilization of antagonistic metabolite-producing rhizosphere and endophytic bacteria, fungi and endophytes, organic residues, and diverse soil amendments (Yuliar and Toyota, 2015). However, many of these control strategies prove ineffective against the biofilm forms of growth, which significantly contribute to the virulence of the phytopathogen.

Phage therapy utilizing lytic phages, emerges as a promising alternative for controlling pathogenic bacteria (Abedon *et al*., 2017; Svircev *et al*., 2018). These lytic phages can selectively and effectively lyse their bacterial targets without impacting the surrounding microbiota. During treatment, they are self-

replicating and self-limiting, providing a safe natural product with relatively low production costs (Abedon *et al*., 2017). The potential of phages to control significant plant diseases has already been proposed, underlining their importance in this field (Jones *et al*., 2007; Balogh *et al*., 2010; Svircev *et al*., 2018). Bacterial wilt biocontrol utilizing bacteriophages has been explored, against strains belonging to *R.solanaceraum* (Yamada *et al*., 2007; Fujiwara *et al*., 2011; Bae *et al*., 2012; Kalpage and De Costa, 2014; Bhunchoth *et al*., 2015; Wei *et al*., 2017, Alvarez *et al*., 2019; Umrao *et al*., 2021).

In this study, therefore, we isolated two novel phages infecting *R.solanaceraum* isolates from environmental water and soils and characterized to use them as biological control entities.

Materials and methods

Bacterial isolates: In this study, strains of *R. solanaceraum* were cultivated in Casamino acid, peptone, and glucose (CPG) medium as well as Nutrient Broth (NB), at a temperature of 28°C. Pathogenicity was confirmed on susceptible varieties of tomato and brinjal. Additionally, the bacterial isolates were identified as *R. solanacearum* by amplifying the fliC and Rs-sp loci using polymerase chain reaction (PCR) with specific primers (F- "GAACGCCAACGGTGCGAACT", R-"GGCGGCCTTCAGGGAGGTC" for fliC; F-"GTCGCCGTCAACTCACTTTCC", R-"GTCGCCGTCAGCAATGCGGAATC" for Rs-sp).

Phage isolation: Soil samples were gathered from tomato and brinjal fields in Northern Karnataka. Each 10 g of soil was mixed with 50 mL of sterile distilled water and vigorously agitated for 20 min. at room temperature to release bacteriophages. For pond water or municipal sewage water, an equal volume of phage broth and 1 mL of broth from a 24-hour-old culture of *R. solanacearum* bacteria were combined, followed by 24 hours of incubation. Subsequently, the mixtures were centrifuged at 10,000 rpm for 15 min. to separate the supernatant and filtered through 0.22 µm pore size filters to eliminate bacteria. The plaque forming units (PFU) assay used the double-layer agar diffusion method. Phages were subsequently purified through three successive single plaque isolations using phage lysate buffer (SM buffer) and stored in plastic vials at 4°C in complete darkness.

Determination of host range of phages: To evaluate the efficacy of isolated bacteriophages, they were plated against various isolated *R. solanacearum* bacteria, including both pathogenic and non-pathogenic strains such as *Escherichia coli, Bacillus subtilis, Pseudomonas fluorescence, Xanthomonas axonopodis* pv. *malvacearum, Ralstonia solanacearum*, and *Xanthomonas axonopodis* pv. *citri*. This assessment utilized the double agar overlay assay to determine the phage's ability to form plaques on the bacterial lawn. The test involved simultaneous plating of a phage suspension with a known concentration on both the test strain and the host strain. The Efficiency of Plating (EOP) was calculated by dividing the number of plaques observed on the test strain by the number of plaques on the host strain. A test strain was considered sensitive if the EOP was ≥ 0.1, while a strain was deemed resistant if the EOP was less than 0.01.

Assay for chloroform sensitivity and temperature stability: In the initial step, optimal phage dilutions of 10^5 PFU/mL were treated with 5% (v/v) chloroform. Following this treatment, the suspension was gently agitated at 100 rpm at room temperature (25±1°C) for 15 min.. Subsequently, the suspension underwent centrifugation at 10,000 g for 10 min. at 4°C. Finally, the phage titration was conducted using the standard double-layer plating method after a 12-hour incubation period. For the thermal stability assay, optimal phage dilutions of 10^5 PFU/mL were prepared in phage buffer. Diluted phages were exposed to temperatures ranging from 45°C to 70°C. At specified time intervals (0 min - control at room temperature, 5, 10, 20, 30, 40, 50, and 60 min), 100 µL aliquots were removed and plated using the double-layer plating technique with an overnight culture of the host bacterium. Phage diluted in phage buffer served as a control. All plates were then incubated at 37°C for 12 hours, after which the number of plaques was counted and the phage titre was calculated for each time interval and its associated temperature.

One-step growth curve: To quantify and monitor phage growth (ϕRspv2). in an *R. solanacearum* host, we adopted following method: Initially, bacteria were briefly incubated with a single phage. Subsequently, the mixture was significantly diluted to reduce the bacteria available for phage adsorption. In triplicate microfuge tubes, 1 mL aliquots of bacterial cultures and the respective phage stock were added, aiming for a multiplicity of infection (MOI) of approximately 0.01. This resulted in final concentrations of about 5×10^8 CFU/mL and 5×10^6 PFU/mL. The mixture was then incubated for 10 min. to facilitate phage adsorption to the host cells. Following incubation, the mixture was subjected to centrifugation at 6000g for 10 min., and the supernatant was removed to eliminate unadsorbed viruses. The pellet containing the bacteria was resuspended in 1 mL of liquid medium. These steps were repeated three times to ensure the removal of any further unadsorbed phages. Subsequently, 50 µL of the resuspended culture was added to a 50 mL growth medium and incubated at 37°C. A plaque assay determined the number of plaque-forming units (PFU). The burst size, representing the number of phages produced from each infected centre, was determined by fitting sigmoid curves to the data for the best fit..

Phage morphology by transmission electron microscopy: One-fourth of the volume of a 20% PEG 8000, 2.5M NaCl solution was added to the phage lysate to precipitate phages. This mixture was then incubated on ice for 1 h before being centrifuged at 10,000 rpm for 15 min. at 4°C. Following centrifugation, we discarded the supernatant and resuspended the pellet in 50 µL of phage buffer (SM buffer). For Transmission Electron Microscopy (TEM) analysis, imaging was conducted at the Electron Microscopy Labouratory of the Virology Division, Indian Agricultural Research Institute, New Delhi. The phages were observed and photographed using a Zeiss EM-10CA transmission electron microscope operating at 100 kV.

Molecular approach for the distinctiveness of bacteriophages

Phage DNA Isolation: To isolate and purify total DNA from bacteriophages using Norgen's Biotek Phage DNA Isolation Kit (Product # 46800), the following steps were performed: 1) One mL of the phage lysate (> 1 x 10^8 PFU/mL) was initially treated with DNAse to prevent host genomic DNA contamination. Subsequently, DNAse-I inactivation was carried out at 75°C for 5 min.. (2) Following DNAse treatment, 500 µL of Lysis Buffer was added to the lysate, and the mixture was incubated at 65°C for 15 min. (3)To precipitate the DNA, 320 µL of isopropanol was introduced to the lysate, and the mixture was centrifuged for

1 min. at 6,000 g in a spin column. (4) The flow-through from the previous step was discarded, and the column was subjected to three washes with 400 µL of Wash Solution, each wash lasting 1 min. and being conducted at 6,000 g. (5) Finally, the DNA was eluted from the column by adding 75 µL of Elution Buffer and then centrifuging for 1 min. at 6,000 g.

Random Amplified Polymorphic DNA (RAPD) analysis of Phage DNA

To know the diversity isolated phages four random primers *viz.*, OPF-02 & OPF-07 and OPB-05 &OPB-11 were used to amplify the phage DNA. Reaction mixture consisting of Template DNA (25 ng/L): 1.0 µL, 10 x assay buffer with 15 mM $MgCl_2$: 2.0 µL**,** dNTPs mix (10 mM): 1.0 µL**,** Random Primer (5 pM/ µL): 1.0 µL, Sterile distilled water: 14.50 µL**,** Taq DNA polymerase (3.0 U/ µL): 0.5 µL was prepared**.** The optimum specifications followed for DNA amplifications are as follows: Initial denaturation at 94°C for 1 min, 40 cycles of denaturation at 94°C for 1 min, primer annealing at 35°C for 1 min and extension at 72°C for 2 min followed by final extension at 72°C for 5 min. Amplified products were separated by 1 per cent agarose gel and documented by Alpha Digidoc 1000 system (Alpha Innotech Corporation, USA).

Results

Bacterial isolates: Six isolates of *R. solanacearum* (RS 1, 2, 3, 4, and 5) were obtained from diseased solanaceous plants, namely brinjal and tomato, and subjected to pathogenicity assessment (Fig 1). Among these isolates, RS3, RS4, RS5, and RS6 exhibited pathogenicity in both tomato and brinjal seedlings. Visible symptoms included the flagging of one or two leaves, primarily observed at the apical regions of the plants. Subsequently, affected plants succumbed to mortality within a remarkably short timeframe, typically within 4-7 days. Molecular characterization involved using a universal Rs-specific primer targeting DNA sequences of 280 base pairs. The amplification success rate indicated that five isolates were indeed *R. solanacearum*. In contrast, one isolate, RS2, failed to amplify, thus indicating its divergence from *R. solanacearum* and precluding its inclusion in subsequent analyses (Fig 2). Furthermore, the virulence nature of the five isolates also confirmed through the amplification of *fliC* loci.

Isolation bacteriophages: Water and soil samples were surveyed to isolate phages targeting *Ralstonia solanacearum*. Notably, 30% of the water samples (nine out of thirty) exhibited lysis activity against *R. solanacearum*. Additionally, soil samples collected from brinjal and tomato fields afflicted with bacterial

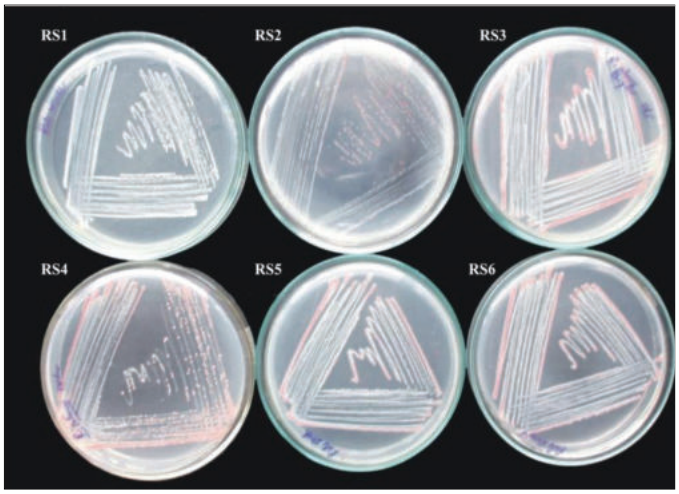
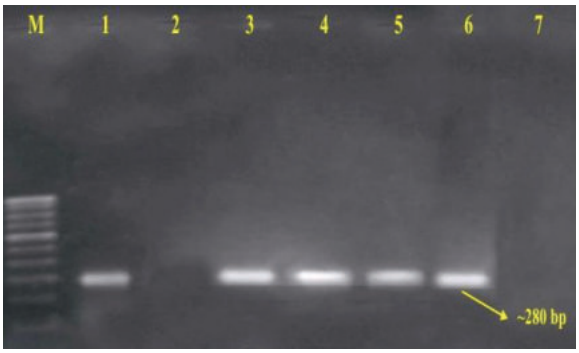
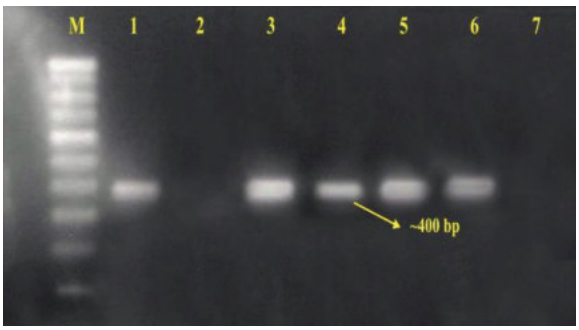


Fig 1. Isolates of *R. solanacearum* on Tetrazolium chloride Agar medium



Genomic DNA amplification of *R. solanacearum* using *Rs-sp* primer pair Lane (M): DNA marker (l00bp). Lanel. 3, 4, 5, 6: amplified product of five isolates RS 1, RS3. RS4, RS5 and RS6, Lane2: nonamplified product of RS2 isolate. 7: Buffer control



Genomic DNA amplification of *R. solanacearum* using *fliC* primer pair. Lane (M): DNA marker (100bp) Lane 1,3,4,5,6: amplified product of five isolates RS 1, RS3, RS4, RS5 and RS6, Lane 2 monamplified product of RS2 isolate, 7: Buffer control.

Fig 2. Molecular confirmation of *R. solanacearum* isolates by PCR

Table 1. Table showing the details of properties and host range of three bacteriophages isolated against *R. solanacearum*

Phage type	Plaque morphology & size (mm) of host strain	Average titre (PFU/mL)	Chloroform sensitivity	Phage morphology	Phage burst size (PFU/mL)	Bacterial strain	EOP	Plaque morphology
ΦRspv1	Clear 2.5-4.0	$3.5x10^3$	Positive	Icosahedral head (55±2 nm) long contractile tail (220±20 nm) (*Myoviridae*)	NA	RS1	0.00	-
						RS3	0.57	Medium, clear
						RS4	0.00	-
						RS 5	0.94	Small, turbid
						RS 6 (Host strain)	1.00	Large, clear
ΦRspv2	Clear 1.0-2.0	$5.8x\ 10^3$	Positive	long fibrous shape of 1300±300 nm in length (*Inoviridae*)	85	RS1	0.00	-
						RS3	0.00	-
						RS4	0.78	Medium, clear
						RS 5	0.00	-
						RS 6	1.00	Small, clear

wilt displayed phage activity at 33%, contrasting with the lower activity observed in uninfected fields. Among the isolated phages, two, named ΦRspv1 (*Ralstonia solanacearum* phage variant 1) and ΦRspv2, were selected for further investigation. Clear plaque morphologies were observed after testing these phages against the RS6 isolate of *R. solanacearum*. The plaque sizes ranged from 2.5-4.0 mm for ΦRspv1 and 1.0-2.0 mm for ΦRspv2 (Table 1 and Fig. 3).

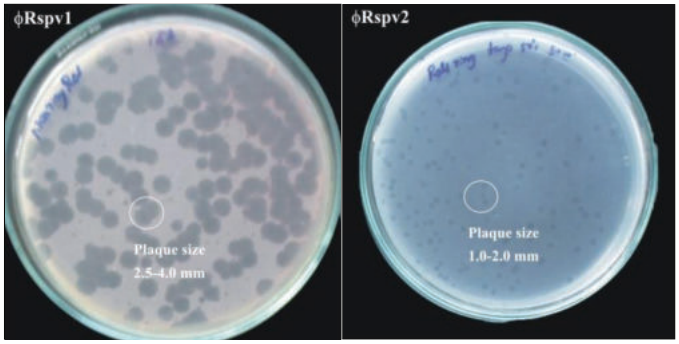


Fig. 3. Plaque characteristics on *R. saolanacearum*

Determination of host range studies: The two bacteriophage isolates demonstrated a heterogeneous lytic pattern when tested against five isolates of *R. solanacearum*. Specifically, ΦRspv1 exhibited distinct plaque morphologies and lytic activity against RS6 (resulting in clear and larger plaques), RS5 (smaller turbid plaques), and RS3 (medium and clear plaques). The efficiency of plating (EOP) values of 0.57 and 0.94 were observed on RS3 and RS5, respectively, indicating high lytic activity (Table 1). On the other hand, ΦRspv2 selectively lysed only the RS4 isolate, producing medium clear plaques with an EOP of 0.784. This suggests that ΦRspv1 possesses a broader lytic spectrum compared to ΦRspv2. In addition, the lytic activity of *R. solanacearum* phages was evaluated against *Escherichia coli, Bacillus subtilis, Pseudomonas fluorescence*, and *Xanthomonas axonopodis* pv. *malvacearum*, as well as *Xanthomonas axonopodis* pv. *citri*. No lytic activity was observed against these bacterial species except within their respective host strains. This specificity indicates a low risk of harm to beneficial soil microflora when utilizing these phages as biocontrol agents against *R. solanacearum.*

Assay for chloroform sensitivity and temperature stability: ΦRspv1 and ΦRspv2, demonstrated insensitivity to chloroform, exhibiting no significant change in titres compared to the control (Table 1). This resistance suggests a probable absence of lipids in

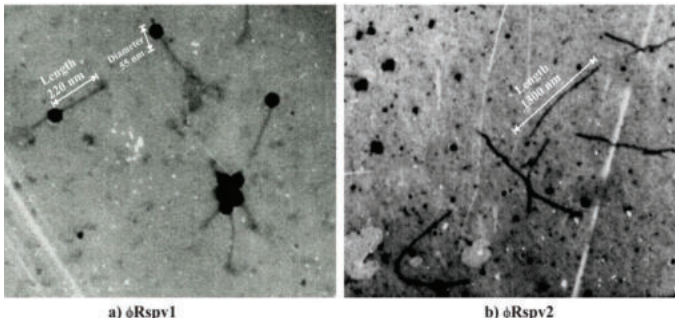


Fig. 4. Particle Morphology of *R. saolanacearum* phages

their composition, rendering chloroform a viable option for large-scale phage preparations in management studies. The impact of temperature on phage viability was investigated at 50°C, 60°C, and 70°C. ΦRspv1 retained 90% viability when exposed to 50°C for 60 min.; however, phage lytic activity sharply declined after that, and no activity was observed at 60-70°C. In contrast, ΦRspv2 exhibited prolonged viability up to 60°C for 20 min., retaining 80% of the control population.

One-step growth curve: The replication dynamics of ΦRspv2 were characterized by a latent period of 60 min., during which no significant increase in phage titer was observed. Following this, a rise period spanning 20-30 min., culminating in a single growth cycle lasting approximately 90 min.. The burst size, representing the number of phage particles released per infected bacterial cell, was estimated to be approximately 85 plaque-forming units (PFU) per cell (Fig 5).

Phage morphology by transmission electron microscopy: Two phages, namely ΦRspv1 and ΦRspv2, exhibited distinct morphologies under transmission electron microscopy (TEM) examination.ΦRspv1 displayed morphological characteristics

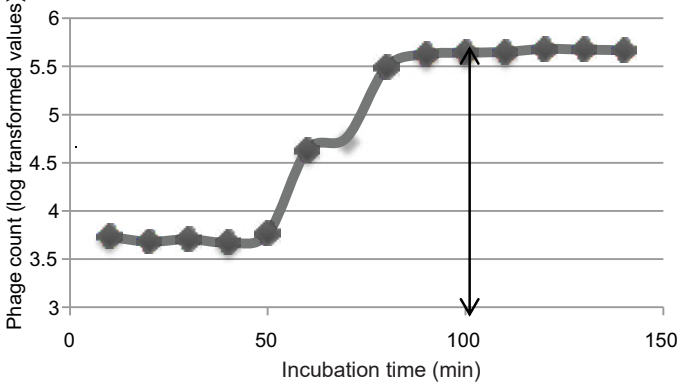


Fig 5. Single step growth curve of ϕRspv2 phage

similar to T_4 phage belonging to the *Myoviridae* family. It featured an icosahedral head with a diameter of 55±2 nm and a long contractile tail measuring 220 ± 20 nm in length. Conversely, ΦRspv2 exhibited a unique morphology resembling the long fibrous shape observed in coliphage M13, associated with the *Inoviridae* family (Fig 4). Its dimensions were measured at 1300±300 nm in length. Bacteriophages in study were classified into their respective families according to the guidelines of the International Committee on Taxonomy of Viruses (ICTV; Ackermann, 2005), offering insights into their genetic and structural characteristics.

Molecular approach for the distinctiveness of bacteriophages: RAPD (Random Amplified Polymorphic DNA) fingerprinting is a cost-effective technique for discerning the genetic diversity within bacteriophage populations. This method is particularly valuable due to the lack of universally conserved genomic regions conducive to traditional diversity analyses. ΦRspv1 and ΦRspv2 phages of *R.solanacearum* were also subjected to RAPD fingerprinting using four random primers and a distinct banding pattern was obtained for each phage (Fig 6). Amplification of DNA was seen for all primers except for ΦRspv1 phage with OPB-11 primer. The amplified profile revealed very poor similarity between ΦRspv1 and ΦRspv2, proving they are genetically distinct.

Discussion

Ralstonia solanacearum, the causal agent of bacterial wilt, is a notorious pathogen affecting numerous economically important

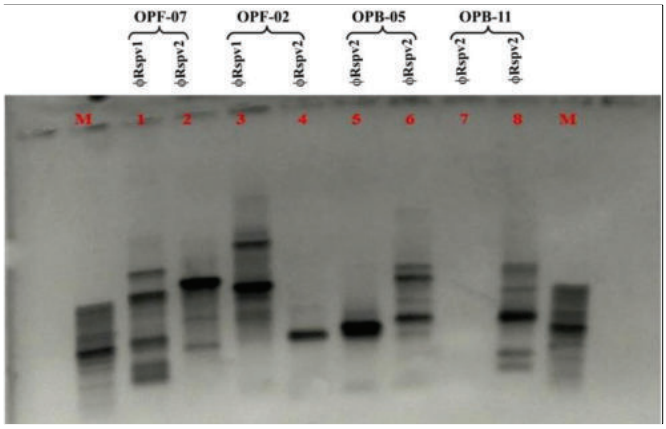


Fig. 6. RAPD profile of *R. saolanacearum* Phages

solanaceous crops worldwide. The pathogen has a wide geographic distribution, affecting developed and developing countries (Genin and Denny, 2012; Prior *et al*., 2012). Its ability to survive in soil and water reservoirs facilitates its spread, posing global agricultural trade and food security challenges. Control of *R. solanacearum* is complex due to its diverse host range, genetic variability, and ability to develop resistance to chemical and biological control measures (Wang and Zhang, 2019). In recent times, there has been a resurgence of interest among agricultural researchers in phage therapy, evidenced by recent scholarly works. Notably, phage-based biocontrol investigations now concentrate on combating the most prevalent and detrimental plant pathogens (Buttimer *et al*., 2017).

Initially, five isolates of *R. solanacearum* showing fluidal, irregular, white or white with pink centers colonies, typical for *R.solanaceraum* virulent form, were confirmed for their pathogenicity along with molecular confirmation was done using species-specific primers 282 bp band. Flagellin, encoded by the *fliC* gene, is the essential subunit of the flagellar filament that is needed for invasive virulence (Tans-Kersten *et al.,* 2001). These primers were used for *R.solanaceraum* confirmation in various studies (Castillo and Greenberg, 2007; li *et al.,* 2014; Sapna and Ramesh, 2014).

The current study investigated the lytic activity of two phages targeting *R. solanacearum*, a causative agent of bacterial wilt in solanaceous crops. Among the collected water samples, 30% (nine out of thirty) exhibited lysis activity against *R. solanacearum*. Similarly, soil samples obtained from fields affected by bacterial wilt in brinjal and tomato crops showed phage activity in 33% (two out of six samples) of cases. In contrast, no such activity was observed in samples from uninfected fields. These findings align with previous research by Yamada *et al*. (2007), Fujiwara *et al*. (2011), Kalpage and De Costa (2014), and Bhunchoth *et al*. (2015), which also demonstrated the presence of bacteriophages targeting *R. solanacearum* in soil samples from areas afflicted by bacterial wilt. Phage preparations have been utilized for controlling various bacterial plant pathogens, including *R. solanacearum* (Ramírez *et al*., 2020), *Xanthomonas spp.* (Tewfike and Desoky, 2015; Ibrahim *et al*., 2017), *Xanthomonas oryzae* pv *oryzae* (Ranjani *et al*., 2018; Jain *et al*., 2023) and *Pectobacterium carotovorum* subsp. *carotovorum* (Zaczek-Moczydłowska *et al*., 2020). Researchers have consistently found these phage preparations effective in limiting disease incidence and severity.

In our results, ΦRspv1 phage lysed three isolates of *R. solanacearum* with distinct plaque morphology. EOP was more than 0.1, showing broad lytic activity. But, ΦRspv2 lysed only one isolate of *R. solanacearum* that produced larger clear plaques with EOP of 0.784. It shows ΦRspv1 has higher broad lytic activity than ΦRspv2 can be used as a biocontrol agent in phage mixtures. None of the phages could infect the bacteria other than *R. solanacearum,* thus indicating their host specificity. This minimizes the risk of phage attack on beneficial microbes present in the agricultural field if, in the future, it is used as a biocontrol agent against *R. solanacearum*.

ΦRspv1 and ΦRspv2 demonstrated tolerance to chloroform treatment and their lytic ability was not compromised. The presence of chloroform in these experiments had no discernible effect on the phages mentioned above, suggesting the possibility of the absence of lipids in their composition. Consequently, chloroform can be consistently used for large-scale phage preparations in management studies. Temperature stability studies showed that ΦRspv2 phage has relatively long periods of viability up to 60^{o}C for 20 min with 80 per cent of the control population when compared to ΦRspv1 phage of *R. solanacearum* that lost activity after being exposed to 50^{o}C for 40 min. These results correlate with previous studies where phage lytic ability was shown to be reduced by prolonged exposure to high temperatures (Da Silva and Janes, 2005; Bryant *et al.,* 2007; Chandra *et al.,* 2011).

Phages that complete their life cycle in a shorter time with larger burst sizes are generally more suitable as biocontrol agents. The burst size of phage can vary depending on factors such as the host strain, culture medium, culture conditions, cell age, and MOI (Hadas *et al*., 1997). Particle morphology of isolated phages is in agreement with Bhunchoth *et al.* (2015) and Yamada *et al. (*2007), who also observed phages of *R.solanacearum,* exhibiting features of the family *Myoviridae* and *Inoviridae,* respectively.

The Random Amplified Polymorphic DNA (RAPD) PCR analysis stands out as a dependable and cheap method for discerning closely related phages, as evidenced by Jothikumar *et al*. (2000), who employed it to generate fingerprints of 10 isolated phages targeting *Escherichia coli*. This technique has demonstrated versatility, being utilized to evaluate genetic diversity across various phage populations, including virophages (Comeau *et al*., 2006; Shivu *et al*., 2007), phages infecting *E. coli* (Dini and Urraza, 2010), and *Pseudomonas aeruginosa* (Li *et al*., 2010). In the present study, RAPD PCR successfully distinguished between isolated phages, highlighting unique genetic variations.

In conclusion, it sheds light on the potential application of bacteriophages as biocontrol agents. It highlights that phages can serve as effective alternatives to hazardous chemicals. However, evaluating their efficacy under field conditions is essential to determine their potential in disease control. Furthermore, the success of phage therapy hinges on the careful selection and mixing of phagovars with a broad host range. This step is crucial in ensuring the effectiveness of phage-based treatments.

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