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ISOLATION AND DNA FINGERPRINTING ANALYSIS OF BACTERIOPHAGES SPECIFIC TO *RALSTONIA SOLANACEARUM*

MAKARI HANUMANTHAPPA K[1*], PALANISWAMY M[2], ANGAYARKANNI J[3] AND VINAY SUVARNA M N[4]

[*1, 4]*Research and Development Centre, Bharathiar University, Coimbatore-641046, Tamil Nadu, INDIA*
[*]*Department of Biotechnology, IDSG Government College, Chikmagalur-577102, Karnataka, INDIA*
[2]*Department of Microbiology, School of Life Sciences, Karpagam University, Coimbatore-641021, Tamil Nadu, INDIA*
[3]*Department of Microbial Biotechnology, Bharathiar University, Coimbatore-641046, Tamil Nadu, INDIA*

ABSTRACT

The aim of this research work was to analyze the genetic diversity of the lytic bacteriophages isolated against phytopathogenic *Ralstonia solanacearum*. The 16S rRNA sequence analysis was used for the identification of the bacteria. Four lytic phages: ϕHMPM12, ϕHMPMA12,ϕHMPMB12 and ϕHMPMC12 specific to *R. solanacearum* were isolated from sewage sample. The phage ϕHMPM12 was found more stable in all temperature ranges. Transmission electron microscopic (TEM) analysis was used to determine the morphology of the phages. Electronic microscopic images of phages revealed that bacteriophages have possessed icosahedral head shape of 45-55 nm in diameter and a non-contractile tail of 210-235 nm in length, these morphological similarities of the phages were represents the family *Siphoviridae*. The RAPD PCR technique was employed with primers OPC04 sequence 5′-CCGCATCTAC-3′ and OPC05 sequence 5′-GATGACCGCC-3′ series. The genomic fingerprinting of all the four phages by RAPD PCR revealed the distinct banding patterns. The findings of the study support the use of RAPD PCR technique for the quick typing of phage isolates and assessment of genetic diversity.

KEY WORDS: **Bacteriophage, *Ralstonia solanacearum*, scanning electron microscopy RAPD-PCR and *Siphoviridae***

MAKARI HANUMANTHAPPA K
Research and Development Centre, Bharathiar University, Coimbatore-641046. Tamil Nadu. INDIA

***Corresponding author**

INTRODUCTION

Ralstonia solanacearum is a Gram-negative phytopathogenic bacteria causing devasting lethality in *solanaceae* family plants. It colonizes the xylem, causing bacterial wilt in a very wide range of potential host plants. *R. solanacearum* is now of the more intensively studied phytopathogenic bacteria and bacterial wilt of tomato is a model system for investigating mechanisms of pathogenesis. *R. solanacearum* is classified as one of the world's most important phytopathogenic bacteria due to its lethality, persistence, wide host range and broad geographic distribution. Although the pathogen causes major yield lost in the tropics and subtropics, it is currently a continuing threat in temperate climates [1]. *Pseudomonas solanacearum* (Smith) is the causal agent of brown rot of potato and bacterial wilt in potato, tomato and other *solanaceae* species [2]. The plants become withered and produce small fruits of poor quality. As a result, yields are severely affected leading to commercial losses for growers. Chemical and cultural control of this disease in infested soils is a hard task [3]. *R. solanacearum* is a soil borne plant-pathogenic β- proteobacterium with a wide host range and geographic distribution. Plants in at least 54 botanical families are affected in tropical and subtropical midland regions of the world [2, 4, 5]. Crop rotation using resistant varieties was considered as an effective alternative, but the large number of hosts has reduced its effectiveness. The characterization of *R. solanacearum* strains of *Solanaceae* crops were reported in Ethiopia, in this study eighty one isolates of *R. solanacearum* like bacteria on triphenyl tetrazolium chloride (TTC) medium were collected from different *Solanaceae* crops (i.e. potato, tomato and pepper plants and potato tubers) at various sites in Ethiopia [6]. Recently one of the study anticipated the use of alternative bacteriophage mediated control of bacteria. The three phages ϕRSA1, ϕRSB1, and ϕRSL1 appear to be useful in the eradication of the bacterial wilt pathogen[7]. The findings of the research on isolation of lytic bacteriophage against *R. solanacearum* was anticipated the use of phages as biocontrol agent in plant protection[8]. Bacteriophages are the natural killers of bacteria and they kill specific bacteria either by lytic or lysogeneic mode of infections. Bacteriophages also called as viruses of prokaryotes, acts as natural bacterial killers, that infects bacteria and can either instantly kill a bacterial cell. Although phage typing was discovered during the second decade of the 20th century, a widespread appreciation of their viral character of specific bacteria was being used in as biocontrol agents in plant protection [9]. The genomes of phage have either DNA or RNA with many shapes. This DNA or RNA genome is encapsulated in a protein coat, which is connected to a base plate by a tail. An investigation was done to eliminate bacteria as far back as 1921, including *Staphylococci* in human infections [10]. The isolation and RAPD–PCR analysis of bacteriophages against *Pseudomonas aeruginosa* was done to understand morphological features and the genomes of phages [11]. Bacterial infections in agricultural crops and its control is a serious problem since its high multiplication rate, adaptability and propagation inside the plant tissue make them unsuitable and inaccessible for most of the control measures. Bacteriophage mediated control of plant bacterial disease is a fast expanding area and now it is prevalent over chemical control. Several research reports have shown that phages can be used as a very efficient tool for control of phytopathogenic bacteria. Bacteriophages acts as natural enemies of bacteria. The abundant availability and use of simplified protocols for its isolation and characterization influenced greatly to make them as potent antimicrobial agents. Phages are highly specific to the particular host bacteria; environmental friendly nature of phages provides potent biocontrol agents over other bactericides. Based on the available data and reviews, we have attempted the molecular characterization of lytic bacteriophages against phytopathogenic bacteria.

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MATERIALS AND METHODS

Isolation of phytopathogenic bacteria from various infected potato and ginger plants

The bacteria were isolated from wilt infected potato and ginger plants collected from Chikmagalur and Hassan districts, Karnataka from 2009 to 2012 by standard procedure on CPG (10g peptone, 1g cassamino acid, 15g agar in 1000 mL distilled water and added 5 mL of 1% of stock solution of 2,3,5-triphenyltetrazolium chloride (TTC) (after cooling the medium to 55°C) and modified SMSA (10g peptone, 5 mL glycerol, 1g casamino acid, 15g agar, 1000 mL distilled water, 25 mg bacitracin, 100 mg polymyxin ß sulfate, 5.0 mg chloramphenicol, 0.5 mg penicillin G, 5.0 mg crystal violet and 50.0 mg TTC) media were used for the isolation of *Ralstonia solanacearum* from plant material. Simple random technique was adopted for the collection of samples field diagnosis of diseased plant sample was done by critically observing the bacterial with symptoms. Beside *R. solanacearum* infections in potato and ginger crops, all other isolated bacterial types from these plants were characterized by 16S rRNA partial sequence analysis. Bacterial ooze from the plant sample was diluted up to 10^{-8} and 100 µL of oozed suspension was taken from different dilutions (10^{-6}, 10^{-7} and 10^{-8}) poured on the media and spread uniformly by using L-shaped glass rods. The inoculated plates were incubated for 72 h at 28°C [12, 13]. The simple random method was used for the collection of infected potato and ginger plants. Plant materials were collected by critically observing the wilting symptoms. The direct isolation of *R. solanacearum* was obtained from plant ooze and exudates.

Use of specific media

The 2, 3, 5-tripenyl tetrazolium chloride (TZC) media was used for the screening of *R. solanacearum* [14, 15]. Fluidal pinkish red centered colonies of typical *R. solanacearum* were observed on TZC media. The pink colored colonies were picked and purified for confirmation of bacterial wilt causing pathogen.

Genomic DNA extraction and PCR based amplification of 16S rRNA sequences

In the present study, 12 phytopathogenic bacterial strains were screened from different taluk of Hassan District, Karnataka. Among them, four strains were identified as *R. solanacearum* strains.The bacterial isolates were subjected to 16S rRNA sequences analysis.The genomic DNA of *R. solanacearum* was isolated for PCR amplification was done according to the instructional manual provided by Aristogene Biosciences Pvt Ltd, Bangalore, India, (Aristogene PCR kit, 16S rRNA sequence amplification kit, Aristogene Biosciences Pvt Ltd, Bangalore). The genomic DNA was isolated by the CTAB method and subjected to PCR amplification using 16S rRNA F8U universal primers (16s rRNA F-5'AGA GTTTGATCCTGGCTCAG3'; 16S rRNA R-5'ACGGCTACCTTGTTA3') PCR amplification of DNA was performed in Effendorf gradient thermal cycler at the suitable conditions for PCR according to the standard procedure [8,16,17] and the PCR amplified products were separated in agarose gel electrophoresis. Electrophoreses gel was observed for DNA bands on a UV trans-illuminator. The results were documented in Alpha imager Gel Doc system. Eluted DNA samples were subjected to Sequence analysis using cycle sequence method and generated sequences were analyzed by BLAST at NCBI.

Isolation of lytic bacteriophages for phytopathogenic R. solanacearum bacteria

The isolation of bacteriophage was done according to the method of [18]. The bacteriophage was isolated from raw water of municipal sewage treatment plant, Hassan, Karnataka. 50 mL of raw sewage water was collected with a few drops of chloroform in a sterile conical flask. Equal volume of lactic phage broth was added and 1ml broth of 24 hour old culture of *R. solanacearum* was incubated at 37ºC for 24 h. After 24 h of incubation, lysate was added with a few drops of chloroform and shaken for about 15 minutes. The mixture was centrifuged at at 10000 rpm for 15 min, the supernatant was filtered through 0.22 µm pore size Acrodisc membrane filters (Pall, German Laboratory) to remove the lysate of bacteria. The phage

particles were subjected to precipitation using 0.5 M NaCl and 5% polyethylene glycol 6000 and resulted phage preparations were stored at 4ºC. The plaque forming (PFU) assay was conducted using double-layer agar diffusion method previously described by Smith and Huggins [16]. All the experiments were conducted in triplicates to ensure phage isolation. To isolate single colonies of phage infected with *R. solanacearum*, single plaque was picked from the PFU assay petri plates and re-plated thrice to confirm the isolation of single phage type [19]. The phage isolated was designated as *R. solanacearum* phage ϕHMPM-12, ϕHMPMA12, ϕHMPMB12 and ϕHMPMC12.

Effect of temperature on phage stability

Effects of the temperature on the stability of isolated phages were done according to the modified protocols mentioned by [7]. The phage preparations of ϕHMPM12 (10^9 PFU/ml), ϕHMPMA1 (10^9 PFU/ml), ϕHMPMB12 (10^9 PFU/ml) and ϕHMPMC12 (10^9 PFU/ml) in SM buffer (100 mM sodium chloride, 10 mM of magnesium sulphate and 10 mM Tris-HCl, pH 7.5) was incubated in sealed tubes at 5, 10, 17, 28, 37 and 50^0 C for various periods before the plaque assay with the strain *Rs*1, *Rs*2, *Rs*3 and *Rs*4 as the host. A 10 ml volume of each phage solution was added to 100 g of autoclaved field soil. The phage-soil mixture was divided into five equal amounts and separately filled into 15 ml tubes. After sealing, the tubes were incubated at 5, 10, 17, 28, 37 and 50^0 C for various periods for 3, 6, 9, 12, 15 days intervals and optical density, experiments were performed in triplicates.

Study of bacteriophage morphology by transmission electron microscope

Bacteriophage ϕHMPM12, ϕHMPMA12, ϕHMPMB12 and ϕHMPMC12 solutions were filtered through the Acrodisc membrane filter (0.22 μm pore size) to remove bacterial lysate and biological macromolecules of host bacteria. Phage preparations were washed three times with 0.1M ammonium acetate solution (pH 7.0). Further, phage particles were purified by CsCl- gradient ultra-centrifugation. The retained phage solution was subjected to negative staining [5, 20]. Bacteriophages have great potential and they can use as biocontrol agents for pathogenic bacteria. Phage morphology, transmission electron microscopy of *R. solanacearum* phages were performed as described by [21, 11] with modified protocols. Drops of ultracentrifuged phage samples (10,000 rpm for 2hr, 4^0C) were dropped on nitrocellulose coated grids (diameter, 3mm; 300 meshes). After 5 min, the phage particles were stained with 2% (w/v) potassium phosphotungstate (pH 6.8-7.2) for 10s. The grids were allowed to dry for 20 min and examined under a transmission electron microscope.

RAPD analysis of lytic bacteriophages

The genetic diversity of isolated phages were analyzed by Randomly Amplified Polymorphic DNA (RAPD) with primers OPC04 sequence 5′-CCGCATCTAC-3′ and OPC05 sequence 5′-GATGACCGCC-3′ series (Aristogene Biosciences, Ltd, Bangalore, India). Single plaque from each plate was lifted with the help of wide bore pipette tip, labeled respectively and stored with 0.5 ml LB medium at 4ºC. Two to three ml of LB broth was added to Petri plate which has phage plaque and it was kept to stand for 1 hr. Medium with phage was transferred completely into new 2 ml vial and respectively labeled. To this 0.1ml of chloroform was added to lyse the host cells. Kept for 30min and centrifuged for 5min at 10000 rpm. The supernatant was transferred to new vial and stored at 4ºC. The samples of phages used for DNA isolation were suspended in 200μl of LB broth, and kept for 30mins.After 30 mins four suspended samples, four phage samples from plate lysate and Aristogene phage T4 phage ampules were kept at boiling water for 10mins.Then the above samples were diluted (1:1) with distilled water. Meanwhile, RAPD master mix was prepared for 270μl as given below. Amplified RAPD PCR products were electrophoresed on 2% agarose gel μg/ml of ethidium bromide [22].

PCR Reaction Mixture

	For 1 reaction	Cocktail	Notes
Double Distilled water	12 µl	120 µl	
2X PCR master mix	15 µl	150 µl	1X Contains 100µM each of dATP, dGTP, dCTP and dTTP. Assay buffer with 15mM $MgCl_2$, 3U/reaction Taq Polymerase.
Random Primer	1 µl	10 µl	10pM used for each reaction
Total Volume	**30 µl x 10**	**300 µl**	

PCR Programme for RAPD

Temperature	Time	No. of cycles
94˚C	2 minutes	1
94˚C	30 seconds	40
45˚C	1 minute	
72˚C	1min 30 sec	
72˚C	7 minutes	1

RESULTS AND DISCUSSION

Use of specific media

Fluidal pinkish red centered colonies of typical *Ralstonia solanacerum* were observed on TTC media typical isolated colonies were picked and purified for confirmation of bacterial wilt causing pathogen *R. solanacearum*.

Extraction of genomic DNA from bacteria and PCR based 16S rRNA sequence analysis

Isolated bacterial strains were characterized by 16s rRNA sequence analysis and sequences were submitted to GenBank of NCBI. GenBank accession numbers has been allotted includes KF752586, KJ018749, KJ018750 and KJ018751 to four *R. solanacearum* stains Rs1, Rs2, Rs3 and Rs4 respectively. The findings of this research greatly anticipated the use of 16S rRNA sequence analysis for the identification of bacterial strains.

Isolation of lytic bacteriophages for phytopathogenic R. solanacearum bacteria

In the present study four bacteriophages were isolated specific to *R. solanacearum* after isolation these phages were screened for their lytic activity on the basis of clear plaque formation therefore four phages exhibiting potent lytic activity with clear plaques (2 to 3 mm in diameter) were selected for further characterization and designated as ϕHMPM12, ϕHMPMA12,ϕHMPMB12 and ϕHMPMC12. Phages ϕHMPM12 are potent omnilytic and lethal to *R. solanacerum* strains. The electron microscopic observations of negatively stained preparations of phages possessed icosahedral head shape of 45-55 nm in diameter and a non-contractile tail of 210-235 nm in length (figure 3) giving a λ-like morphology as described in the previous report [23]. Only a few work has been documented the phages that infect *R. solanacearum* reperents myovirus group [24, 25, 26]. This report also finds relevance with the phytopathogenic *Erwinia carotovora* phages [27, 28] were placed in the *Siphoviridae* virus group. Thus it can be used as bio-control agent *in vitro* and further application of this technique, *in vivo* study has to be done essentially. The findings of this research may be helpful for the identification of phytopathogenic *R. solanacearum* bacteria with advanced molecular tools, new modalities can be framed for its control.

Effect of temperature on phage stability

The effect of temperature on the stability of ϕHMPM12 was illustrated as shown in the graph phage preparations were kept at different temperatures in the range of 5 to 50^0 C, in the presence or absence of soil (Figure 6), all four phages were essentially stable below 28^0 C, Whereas ϕHMPM12 exhibited greater stability compared to other phages at higher temperatures. After 15 days incubation phage ϕHMPM12 has exhibited better efficacy against *Ralstonia solanacearum* in presence of soil also. Phage ϕHMPM12 was survived after 15 days of incubation at 50^0C there was no phages were found after nine day incubation with ϕHMPMB12 and ϕHMPMC12. The stability of phages under different soil temperatures was observed. At low temperatures the titers of all phages expect ϕHMPM12 were decreased, this shows nonspecific absorption of phages to the soil. The phage ϕHMPM12 exhibited better stability in all temperature range. Thus the phage ϕHMPM12 can be the good antimicrobial agent for phytopathogenic *R. solanacearum*.

RAPD PCR amplification for DNA fingerprinting of lytic phages

The DNA fingerprinting analysis isolated phages from the sewage samples as revealed RAPD PCR were shown in the figure 4. The primers generated different banding pattern with all *R. solanacearum* phages. This represents phages are genetically unique and could be distinguished by RAPD PCR. This technique is also been used to assess the genetic diversity of vibriophages [29, 30] and phages infecting *E.coli* [31] and *Pseudomonas aeruginosa* [32]. Many research papers have been reported genetic diversity analysis of bacteriophages. One of the studies revealed *Podoviridae* phages possessed ds DNA as genetic material [33]. RAPD PCR analysis of phage DNA provides simple and reproducible method of genetically variants of closely related phages in each family in another study [34] also used RAPD to make finger print at 10 isolated phages against *E.coli*. A recent RAPD PCR study on *Pseudomonas aeruginosa* specific phages represented in genetic diversity [11]. Based on all the research reports, in this study RAPD PCR analysis can be used as a reliable method to understand genetic diversity of bacteriophages and its typing.

CONCLUSION

This research enabled us to characterize bacteria infecting to potato and ginger crops. All these bacterial strains were characterized by 16s rRNA sequence analysis and sequences were submitted to GenBank of NCBI and accession number has been allotted. The findings of this research greatly anticipated the use of phages as antimicrobial agents to *R. solanacerum* strains. Phage ϕHMPM12 are potent omnilytic and lethal to *R. solanacerum* strains. Thus, it can be used as a bio-control agent *in vitro*. A further application of this technique, *in vivo* study has to be done essentially. The findings of this research may be helpful for the identification of phytopathogenic *R. solanacearum* bacteria with advanced molecular tools, new modalities can be framed for its control.

Figure 1a and 1b: *R.solanacearum* infections and wilting symptoms in potato and ginger crops

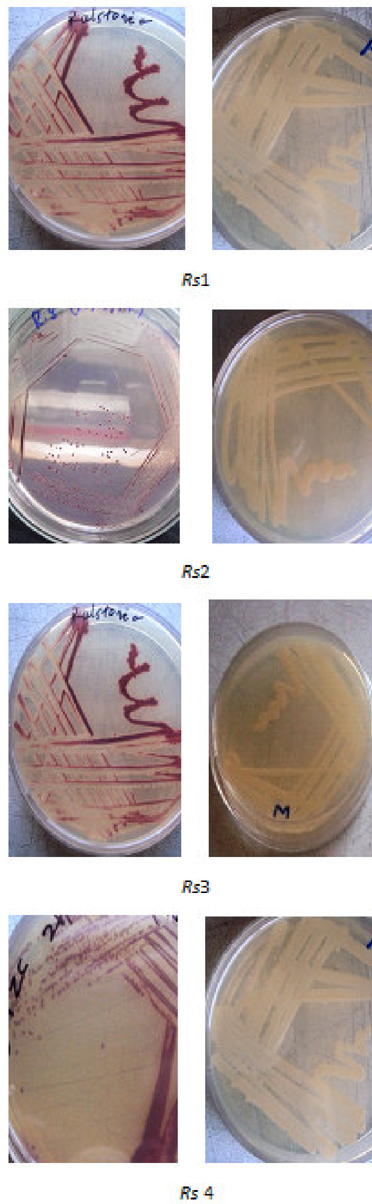

Figure 2: Colonies of *R.solanacearum* on TTC and nutrient agar media

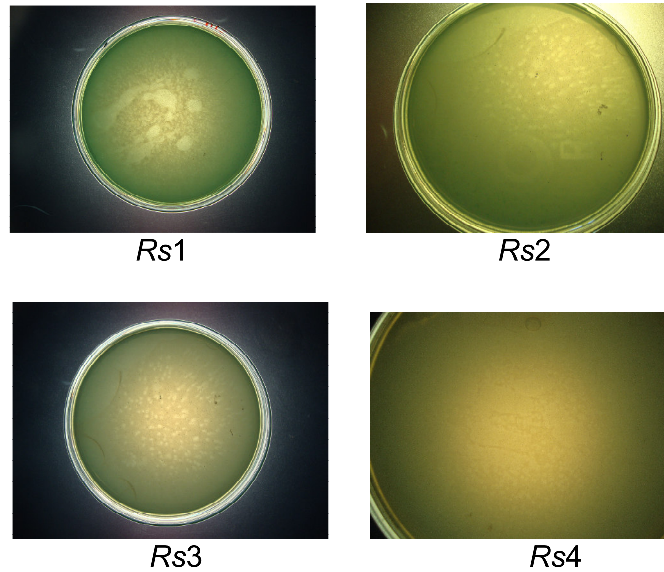

Figure 3
Formation of plaques on Ralstonia solanacearum

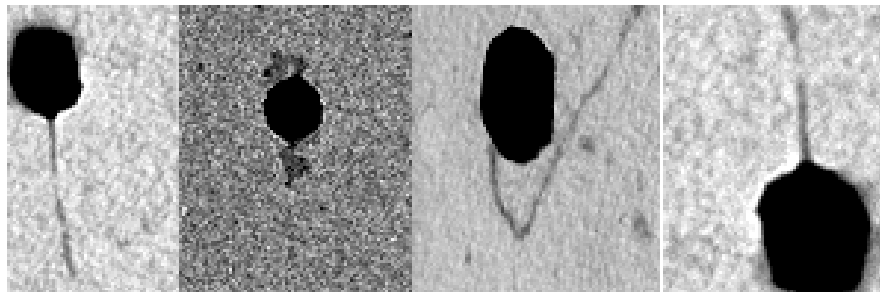

Figure 4
Electron micrographs showing morphology of bacteriophage infecting to R. solanacearum

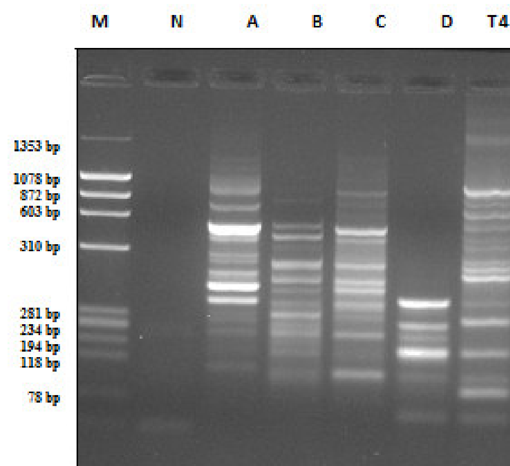

Figure 5
Amplification was seen and polymorphism was observed in all the samples. M: PhiX/Hae marker: sizes 1353, 1078, 872,603,310,281/271, 234, 194, 118, 72 bp, N: Negative control with distilled water, LANE A: ϕHMPM12, LANE B: ϕHMPMA12, LANE C: ϕHMPMB1 and LANE D: ϕHMPMC12. T4: Bacteriophage.

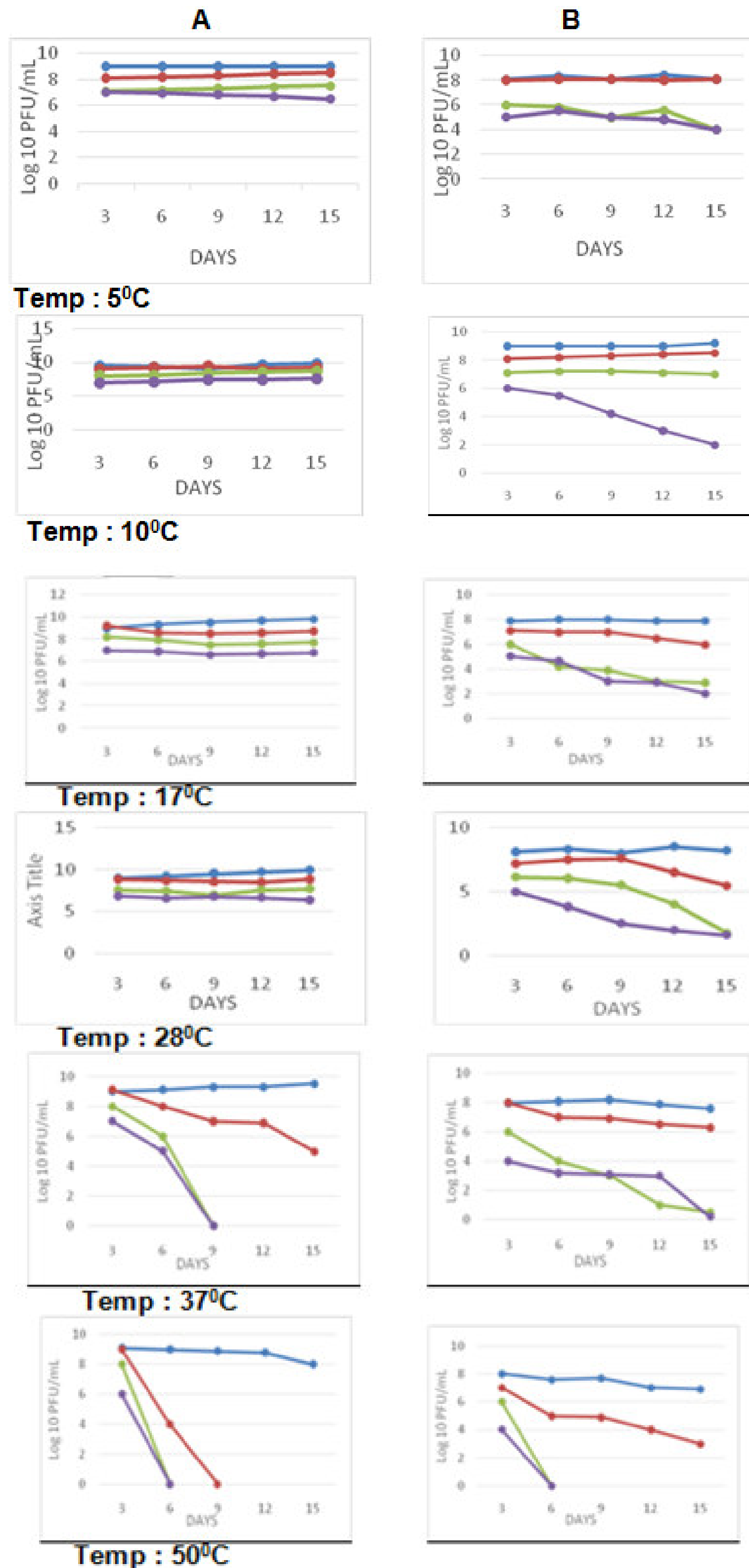

Figure 6

Effect of temperature on the stability of ϕHMPM12 compared with ϕHMPMA12, ϕHMPMB12 and ϕHMPMC12. Phage were kept at a different temperature ranges from 5 to 50^{0} C from various periods without (A) and with (B) soil. ϕHMPM12 exhibited significant stability at higher temperature range.

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