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Isolation of Bacteriophages Infecting *Ralstonia solanacearum* causing Bacterial Wilt Disease in Naga Chilli (*Capsicum chinense* Jacq.)

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A B S T R A C T

Ralstonia solanacearum is a soil inhabiting devastating bacterium causing wilt disease in several important agricultural crops. It has an extremely wide host range and ranked as the 2nd destructive and economically important plant pathogenic bacterium of the world. Although several substances like the antibiotics are used in wide scale for its management. However due to numerous limitations such as the emergence of antibiotic resistant bacterial strains, horizontal gene transfer and so on has led to the re- thinking and diversion of the focus on the use of bio control management strategies. The use of bacteriophages for the control of bacterial diseases is a fast expanding management strategy over chemical control. In the present study, *R. solanacearum* isolates from Naga chilli, chilli pepper, brinjal and banana were isolated and characterized by PCR using specific primers (759/760). Seven lytic bacteriophages were isolated from soil infecting *R. solanacearum* isolate from Naga chilli (F3C2). After three rounds of purification, the characterization of the phages on the basis of plaque morphology and phage titer was carried out. The host range analysis of the isolated phages revealed that these isolated phages were not strain specific but perhaps somewhat race specific in nature. The results of this research shall serve as a basis towards phage based management strategies against the bacterial wilt disease.

Introduction

The bacterial wilt disease caused by *Ralstonia solanacearum*, originally described as *Pseudomonas solanacearum* by Smith (1896), is one of the most devastating bacterial diseases of the world. This phytopathogenic bacterium has a very wide host range and is known to infect more than 200 plant species belonging to 53 different botanical families including several agricultural important crops like potato, tomato, ginger, chilli, banana and others (Alvarez *et al.*, 2010). Because of its exceptionally wide host range and ability to cause disease within a very short time, *R. solanacearum* has been ranked as the 2nd most destructive plant pathogenic bacterium of the world (Mansfield *et al.*, 2012). It is a soil borne gram negative bacterium which is rod shaped with an average size of 0.5 to 0.7 by

1.5 to 2.5 µm. The flagella when present are polar (Denny and Hayward, 2001). *R. solanacearum* has been divided into 5 races in accordance to their ability to infect different host plants.

The race 1 is comprised of many strains having a wide host range and is known to infect various solanaceous and ornamental crops as well as trees and weeds; race 2 is restricted to banana (*Musa* spp.) and *Heliconia* spp.; race 3 also infects solanaceous crops like potato, tomato, brinjal, capsicum and weeds like *Solanum nigram, S. dulcamara* etc.; race 4 infects ginger and race 5 is pathogenic on mulberry (*Morus* spp.) and found only in China (He *et al.,* 1983; Chandrashekara *et al.,* 2012). Apart from races, the strains of *R. solanacearum* is classified into 5 biovars based on its ability to utilize and/or oxidize several hexose alcohols and disaccharides (Hayward, 1964; Hayward, 1991). Based on its phylogeny, the bacterium is divided into 4 phylotypes corresponding to four broad genetic groups; each of them is related to its geographic origin (Alvarez *et al.,* 2010). Direct yield losses by *R. solanacearum* vary widely according to the host, cultivar, climate, soil type, cropping pattern, and strain. The global yield loss ranged from 0 to 91% in tomato, 33 to 90% in potato, 10 to 30% in tobacco, 80 to 100% in banana and up to 20% in groundnut (Yuliar *et al.,* 2015).

Several pesticides such as algicide (3-[3-indolyl] butanoic acid), fumigants (metam sodium, 1, 3-dichloropropene, and chloropicrin) and plant activators generating systemic resistance on the tomato (validamycin A and validoxylamine) have been used to control bacterial wilt. Several antibiotics like triazolothiadiazine, streptomycin sulfate and some other chemicals like bleaching powder or weak acidic electrolyzed water have also shown to effectively destroy the pathogen (Yuliar *et al.,* 2015). The use of antibiotics is the most frequent means of controlling bacterial diseases including bacterial wilt.

However, the indiscriminant targeting of bacteria by broad spectrum antibiotics causes generation of resistance to a wide range of bacterial population. Moreover, non-judicious use of antibiotics has resulted in the spread of R-plasmids and multi-drug resistant (MDR) strains. This has led to re- evaluation and re- thinking of the use of phages as biological control agents (BCA) (Wu *et al.,* 2013). Bacteriophages, commonly known as phages are the viruses that parasitize bacteria. They are ubiquitous and the most abundant organisms found in the nature. The phage therapy or phage based bio control is a fast expanding area of plant protection that has a great potential to replace the prevalent chemical control measures. Apart from being eco- friendly, several other key attributes for instance its ability to break biofilm formation, minimum disruption to micro biota owing to its specificity, effectiveness in handling the problem of antibiotic resistant bacteria, they are considered as potential biocontrol agents. In the recent decade, a great spike in phage therapy research could be observed all over the world. Countries like USA, Japan, China and the European countries play a lead role in the phage therapy research as well as field level application. In India however, it is still in a nascent stage.

There are several recent reports of isolation, characterization as well as *in vivo* successful application of bacteriophages specific to *R. solanacearum*. Yamada (2012) reported four families of bacteriophages namely Myoviridae, Podoviridae, Siphoviridae and Inoviridae that infects *R. solanacearum*. Fujiwara *et al.,* (2011) reported that pretreatment of tomato seedlings with lytic phage ΦRSL1 drastically limited penetration, growth, and movement of root-inoculated *R.*

solanacearum cells. A similar study was carried out by Iriarte *et al.,* (2012) in which they adopted soil based root application and attenuated bacterial strains as a means to improve the persistence of bacteriophages on tomato plants. They reported that the bacterial wilt control was typically best when the phage or phage mixtures were applied to the soil surrounding the tomatoes at the time of inoculation than pre or post inoculation.

Wei *et al.,* (2017) isolated and characterized 12 lytic bacteriophages specific to *R. solanacearum* from potato fields. They reported that 80% of the potato plants could be protected from bacterial wilt using phage cocktail and also reported that the same cocktail could kill 98% of the live bacteria spiked in the sterilized soil one week after application. In India, so far only one bacteriophage (HMPM-2012) was reported to show lytic activity against *R. solanacearum* strains isolated from potato and zinger (Hanumanthappa *et al.,* 2013).

From the above mentioned facts, it can be summarized that in spite of its huge diversity and potential for controlling bacterial diseases, the isolation, characterization and application of phages is very less in India. In the present study therefore, several lytic phages infecting *R. solanacearum* causing bacterial wilt in Naga chilli/ Bhut jolokia were isolated from soil in Assam, India and *in vitro* efficacy was tested on different *R. solanacearum* samples isolated from different host crops.

Materials and Methods

Collection of Samples and Isolation of *Ralstonia solanacearum*

Soil as well as plant samples were collected from various bacterial wilt infected fields in Jorhat District of Assam. Diseased plants showing typical symptoms of bacterial wilt were collected and brought to the laboratory of the Department of Plant Pathology, Assam Agricultural University, Assam, India. The stem and root portions were washed thoroughly under running tap water and surface sterilized with 0.1% $HgCl_2$. For ooze test, a slant cut was given to the stems and immediately dipped in sterile distilled water (dsH_2O). Finally, the ooze was streaked onto plates containing *R. solanacearum* specific Triphenyl Tetrazolium Chloride (TTC) medium (Kelman, 1954). The inoculated plates were incubated at 28 ± 2°C for 48 hours and then stored at 4 °C for subsequent use. For long term storage, 50% glycerol stocks were maintained at -45°C.

Bacterial DNA Extraction

The bacterial DNA was isolated using a method given by Grover *et al.,* (2012) with some modifications. To be specific, *R. solanacearum* isolates were grown in 5ml nutrient broth (NB) for 48 hours at 28 ± 2°C. After incubation, the bacterial cells were pelleted down by centrifugation at 6000rpm for 5 minutes at 4°C. The pellet was suspended in 1ml of dsH_2O, boiled at 100 °C for 5 minutes in a water bath and then vortexed for 2 minutes. Subsequently, the DNA was extracted with phenol: chloroform: isoamyl alcohol (25:24:1) and precipitated by adding 0.1 volume of 5M ammonium acetate and 2 volumes of 100% chilled ethanol. It was then kept in ice for 1 hour followed by centrifugation at 13000rpm for 10 minutes at 4°C. The precipitate obtained was washed with 70% ethanol and then with 100% ethanol and the pellet was air dried and re-suspended in 50 µl of TE buffer. The isolated DNA was stored at -20 °C for further use. The quality and quantity of the extracted DNA was checked by using Eppendorf Bio Spectrophotometer as well as in 1% agarose gel.

Molecular characterization of *R. solanacearum* by PCR analysis using specific primers

PCR based detection of *R. solanacearum* was carried out using specific primers: 759(5´ GTCGCCGTCAACTCACTTTCC 3´)/760 (5´ GTCGC CGTCAGCAATGCGGAATCG 3´) (Opina *et al.,* 1997). Every 10µl of reaction contained 5 µl of EmeraldAmp GT 2x PCR Master Mix, 0.5 µl each of forward and reverse primer (10 pmol concentrations), 1µl of 50ng extracted bacterial DNA and 3 µl of sterile dH_2O. The PCR amplification of the bacterial DNA was carried out in Agilent Technologies Sure Cycler 8800. The **s**amples were initially denatured at 94 °C for 3 mins, annealed at 53 °C for 1 min and extended at 72 °C for 1.5 mins which was followed by 30 cycles of 94 °C for 15 s, 60 °C for 15 s, 72 °C for 15 s, and a final extension of 72 °C for 5 min (Opina *et al.,* 1997). The PCR products were subjected to gel electrophoresis using 1.5% agarose stained with ethidium bromide (0.5µg/ml). The electrophoresis was carried out and visualized under Bio-Rad Gel Doc ™ EZ Imager.

Isolation of bacteriophages infecting *R. solanacearum*

Sampling of soil was carried out from bacterial wilt infected Naga chilli crop fields in Orchard, AAU, Assam, India following previously described protocol with some modification (Bhunchoth *et al.,* 2005). For isolation of phages, 10 g of soil from 5-10 cm depth was taken in a conical flask containing 20 ml Sodium- Magnesium (SM) buffer. The mixture was incubated in an orbital incubator for 24 hours at 28 °C and 180 rpm. The slurry obtained after incubation was centrifuged at 8000 rpm for 20 minutes at 5 °C. The supernatant containing phages was filtered through a 0.2µ size sterile syringe filter containing PVDF membrane (Axiva) to get rid of the inorganic as well as organic debris. Finally, 1 ml of the filtrate is diluted with 9 ml of SM buffer and stored at 4 °C for future use.

For confirmation of the presence of lytic phages, spot test was carried out where initially 500 µl of overnight culture of *R. solanacearum* isolate F3C2 was plated onto TTC plates and allowed to dry under the laminar air flow for around 15 minutes. Phage suspension of 10 µl was then spotted onto the plates and finally the plates were incubated at 28 ± 2°C for 48 hours for observation of lytic spots. The *R. solanacearum* isolate F3C2 was used as a host isolate for the *in vitro* plaque assays as this isolate showed sensitivity to all the isolated phages.

Direct plating of the bacteriophages

The plating of the phages was carried out by direct plating method (Adams, 1959). A serial dilution of the phage stock was carried out by addition of an aliquot of 100 µl from the phage stock to 900 µl of SM buffer in an Eppendorf micro centrifuge tube. Serial dilution was done from 10^{-1} to 10^{-10} dilution. Subsequently, 250 µl of the diluted phages suspension from each dilution was added separately to 250 µl of overnight culture of *R. solanacearum* in a micro centrifuge tube and mixed by inversion. The mixtures were incubated at 37ºC for 20 minutes to allow the phages to be adsorbed to the bacterial cells. After incubation, 500 µl of the mixture was poured on petri dishes containing TTC medium and coated well using the spread plate technique. The plates were then incubated at 28 ± 2°C for 48 hours and finally observed for the presence of plaques.

Purification of the isolated bacteriophages

Direct plating of the isolated phages resulted in the formation of plaques with different

morphology. Hence, the phages were divided into different isolates based on its plaque morphology. Each plaque with a unique morphology was picked individually using a micropipette tip and placed in 10ml of 18- 24 hour old bacterial culture of *R. solanacearum* in NB. The culture was then incubated in an orbital incubator at 28 ± 2°C for 48 hours. The enriched phage lysate was passed through a 0.2µ size sterile syringe filter, diluted in SM buffer as mentioned above and stored at 4°C. A total of 3 rounds of purification were carried out for each phage isolate in order to obtain phages with the same genetic code.

Plaque morphology and phage titer

The plaque size measurement and phage titer of all the purified phages was carried out after 48 hours of plating. A total of 30 plaques were measured for each isolate and the average along with the standard deviation was calculated out. The plaques having core diameter of less than 1.5 mm were considered small. For measurement of phage titer, petri plates bearing a range of 30-300 plaques were used for counting. The average value of the number of plaques for three replications was used.

To determine the titer of the original phage preparation, the following formula was used:
Number of plaques × 10 × Reciprocal of counted dilution ×4 = Plaque forming unit (pfu/ml) (Kropinski *et al.*, 2009) (Since 250 µl of phage suspension was used for plating, hence, multiplied by 4)

Host range analysis of the isolated bacteriophages

In order to determine the broad spectrum ability of the isolated phages, the host range analysis was carried out *in vitro* by using spot test method. The 7 isolated phages were tested against 10 *R. solanacearum* isolates by dotting 10µl of phage suspension over the lawn of *R. solanacearum* and incubated at 28 ± 2°C for 48 hours for observation of lytic spots.

Results and Discussion

Collection of samples for isolation of *Ralstonia solanacearum* and colony morphology in different media

A total of 10 isolates of *R. solanacearum* was collected from wilt infected crop fields in Jorhat District of Assam (Fig. 1a and 1b). The hosts from which *R. solanacearum* was isolated are Naga chilli/ Bhut jolokia (*Capsicum chinense* Jacq.), Chilli pepper (*Capsicum annuum L.*), Brinjal (*Solanum melongena L.*) and Banana (*Musa* sp.). Milky white bacterial ooze was observed coming out of the cut stems of the wilted plants when dipped in dsH_2O (Fig. 2). For isolation of bacteriophages, soil samples were collected from the root zone of wilted Naga chilli crops from a depth of 5-10 cm. All the isolates of *R. solanacearum* produced the typical irregularly-round, fluidal whitish colonies with a pinkish red centre in TTC media (Fig. 3). The same isolates produced white colonies in nutrient agar media. Kelman (1954) reported that the *R. solanacearum* specific TTC media enables the differentiation of virulent isolates from the avirulent and less virulent mutants as the virulent normal or wilt types yielded irregularly-round, fluidal white colonies with a pink center on this medium while the common mutants encountered in stock cultures formed round butyrous (dry) deep-red colonies with a narrow bluish border.

PCR based detection of *Ralstonia solanacearum* using specific primers

In the present study, the PCR based detection of *R. solanacearum* isolates was carried out

using specific primers (759/760). The agarose gel electrophoresis of the PCR amplified product of all the 10 *R. solanacearum* isolates yielded a band size of 281 bp (Fig. 4). No band was observed in negative control. Hence, the PCR results confirmed that the bacterial isolates used in this study were of *R. solanacearum*. Opina *et al.,* (1997) reported that this small portion of the genomic DNA of *R. solanacearum* that is detected by 759/560 primers is a component of a highly conserved region essential for function of all the *R. solanacearum* strains.

Isolation of bacteriophages infecting *R. solanacearum* and *in vitro* plaque assays

Bacteriophages were successfully isolated from the soil samples of bacterial wilt infected Naga chilli/ Bhut jolokia crop field in Orchard, AAU in May, 2018 and stored in SM buffer at 4°C. From the initial sample, 7 phage isolates were separated based on plaque morphology (Table 1) and 3 rounds of purification was carried out. Mullan (2001) mentioned that at least two rounds of purifications should be performed to get single phage-strain population. In the present study, after 3 rounds of purification, phages with same genetic code were obtained. The *R. solanacearum* isolate F3C2 was used for the propagation and study of these 7 phage isolates.

The plaques formed on *R. solanacearum* lawns in TTC media were studied after 48 hours of plating. The plaque morphologies of the 7 isolated phages are shown in Figure 5 (a-g). In the present study, plaques having a core diameter of less than 1.5mm were considered small. Amongst the 7 isolated and purified phage isolates, all the phages except RSP4 had a big and clear core which was surrounded by a halo. The phage isolate RSP4 had a big and clear circular plaque of 2.56±0.41mm size devoid of any halo. The isolates RSP1 and RSP2 had a small core diameter whereas, RSP3, RSP5, RSP6 and RSP7 had a big and core diameter with a translucent halo surrounding the clear core. The phage RSP7 had the largest plaque of 5.41±0.63.

Table.1 Plaque morphology and phage titer of bacteriophages infecting *R. solanacearum*

Sample Name	Plaque Description (After 24 hours of plating)	Plaque Size			Phage Titer (pfu/ml)
		Core Diameter (mm)	Size of Halo (mm)	Size of the complete Plaque (mm)	
RSP1	Very small clear centre with halo	0.26±0.09	0.25±0.06	0.76±0.15	4.8×10^{7}
RSP2	Small centre with halo	0.63±0.13	0.32±0.13	1.27±0.31	2.6×10^{8}
RSP3	Big centre with halo	1.36±0.25	0.61±0.16	2.59±0.39	8×10^{5}
RSP4	Big centre without halo	2.56±0.41		2.56±0.41	7.2×10^{5}
RSP5	Big centre with halo	2.12±0.40	0.60±0.25	3.33±0.42	8×10^{6}
RSP6	Very Big centre with halo	3.11±0.39	0.77±0.34	4.65±0.57	1.2×10^{6}
RSP7	Very Big centre with big halo	4.11±0.42	0.65±0.23	5.41±0.63	2.8×10^{9}

Table.2 Host Range Analysis of the Isolated Phages of R. *solanacearum*

Sl. No.	*R. solanacearum* isolates	Collection Site	Host	Phages						
				RSP1	RSP2	RSP3	RSP4	RSP5	RSP6	RSP7
1	F3C2	Orchard, AAU	Bhoot jolokia (*Capsicum chinense* Jacq.)	+	+	+	+	+	+	+
2	F1C1	Bogoli Sapori, Alangmora, Jorhat	Chilli (*Capsicum annuum* L.)	-	-	-	+	-	-	-
3	F2B1	Upar kareng Village, Jorhat	Brinjal (*Solanum melongena* L.)	-	+	+	+	+	+	+
4	F3C3	Orchard, AAU	Bhoot jolokia	-	-	-	+	-	-	-
5	F3C4	Orchard, AAU	Bhoot jolokia	-	-	-	+	-	-	-
6	F5B1	Gorumora, Jorhat	Brinjal (*Solanum melongena* L.)	-	-	-	-	-	-	-
7	F5B2	Gorumora, Jorhat	Brinjal (*Solanum melongena* L.)	-	+	-	-	-	-	-
8	F1B1	Bogoli Sapori, Alangmora, Jorhat	Brinjal (*Solanum melongena* L.)	-	-	-	-	-	-	-
9	F3C1	Orchard, AAU	Bhoot jolokia (*Capsicum chinense* Jacq.)	-	-	-	-	-	-	-
10	F7BN1	Koronga, Jorhat	Banana (*Musa* sp.)	-	-	-	-	-	-	-

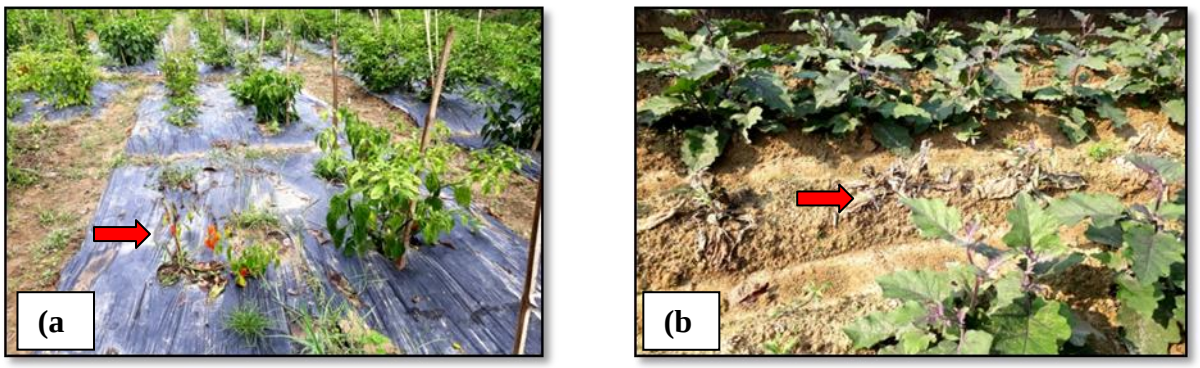


Fig.1 Wilting Symptoms of *R. solanacearum* infected plants shown in (a) Bhut Jolokia/ Naga Chilli (*Capsicum chinense*) and (b) Brinjal/Egg plant (*Solanum melongena*)

Fig.2 Milky white bacterial ooze coming out of cut stem of brinjal infected with *R. solanacearum*

Fig.3 *R. solanacearum* exhibiting irregularly round, fluidal whitish colonies with a pinkish red centre in TTC media

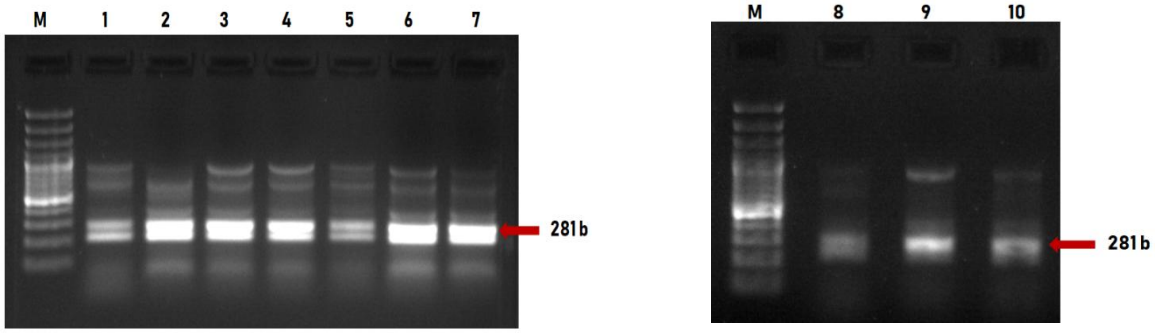
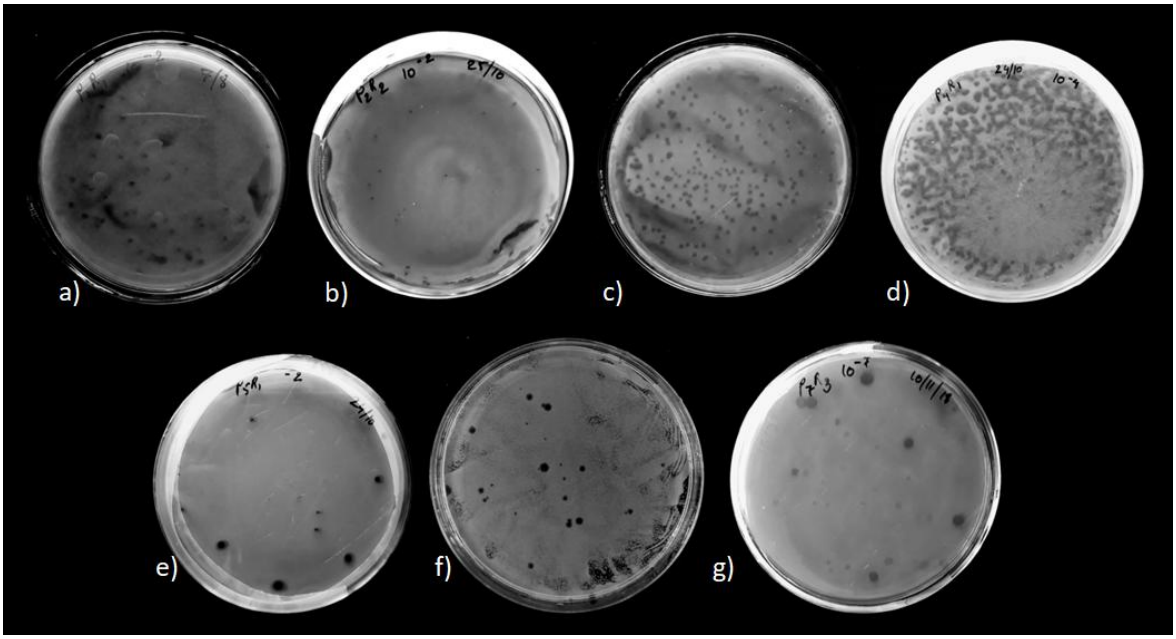


Fig.4 The agarose gel electrophoresis of the PCR amplified product of 10 *R. Solanacearum* isolates showing band size of 281b. Lane M: = 100BP DNA Ladder; Lane 1 to 10: *R. Solanacearum* isolates F3C2, F1CI, F2B1, F3C3, F3C4, F5B1, F5B2, FIBI, F3C1 and F7BN1 respectively.

Fig.5 Plaque morphologies of the 7 isolated phage samples. a) Phage RSP1, b) Phage RSP2, c) Phage RSP3, d) Phage RSP4, e) Phage RSP5, f) Phage RSP6 and g) Phage RSP7



This phenomenon has been described by Abedon and Yin (2009) as within plaque inhomogeneity i.e. the turbidity was seen surrounding the central zone of clearing and is the result of retention of intact bacteria within the plaque clearing. The phage titer was also calculated after 48 hours of plating where the plates having 30-300 plaques were used for counting. Amid the phage isolates, the phage isolate RSP7 had the highest phage titer of 2.8 x 10^9 pfu/ml followed by RSP2 with a titer of 2.6 x 10^8 pfu/ml. However, the phage isolates RSP3 and RSP4 had a considerably low titer of 8 x 10^5 pfu/ml and 7.2 x 10^5 pfu/ml respectively. It was observed that the phage isolate RSP7 had both the largest plaque size as well as the highest phage titer. The differences between the plaque morphology and phage titer occur due to variations in the rate of adsorption, lysis time or the size of virion (Gallet *et al.*, 2011)

Host range analysis of the isolated phages

The host range analysis was carried out to determine the broad spectrum ability of the isolated phages. The 10 isolates of *R. solanacearum* were tested against the 7 isolated phages by dotting assay to observe the formation of lytic zones in lawns of *R. solanacearum* in TTC plates (Table 2). The phage RSP4 showed a relatively broad host range by infecting 5 out of 10 *R. solanacearum* isolates isolated from Naga chilli, brinjal and chilli pepper which was followed by RSP2 infecting 3 *R. solanacearum* isolates. The *R. solanacearum* isolate F3C2 was taken as the host isolate as it was found susceptible to all the 7 phages. The brinjal isolate F2B1 also showed a high degree of susceptibility to all the test phages except RSP1. It was also observed that none of the phages were susceptible against the *R. solanacearum* isolate F7BN1 which was isolated from banana causing moko disease. The host range studies indicated that these phages were somewhat race specific but not strain specific in nature.

In conclusion, the objective of the present research was to isolate and partially characterize the bacteriophages infecting *R.*

solanacearum from Jorhat District of Assam, India. This is the first report of *R. solanacearum*-infecting phages from Bhut jolokia/ Naga chilli crop from India. The results obtained from this study will contribute towards further research on characterization and utilization of these lytic phages as a potential bio control agent for the management of bacterial wilt disease in the near future.

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