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Mitigation of the Tomato Bacterial Speck Pathogen *Pseudomonas syringae* pv. *tomato* by Phage Therapy

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A B S T R A C T

Pseudomonas syringae is one of the predominant bacteria that cause pathogenic infections in a wide variety of fruits, vegetables and ornamental plants. This bacterium produces the toxin coronatine which force the plant to keep its stomata open through which it enters the plant. This infection gradually reduces the plant yield resulting in a great economic loss. Tomato (*Solanum lycopersicum)* plants infected by the bacterial speck pathogen *Pseudomonas syringae* pv. *tomato* was identified by the appearance of the lesions on the leaves and fruits. The pathogen was isolated from the collected samples and confirmed after performing biochemical tests. Phages that target this pathogen were isolated by suspending the collected samples in PBS followed by double agar overlay method. Isolated phages were subjected to purification using polyethylene glycol (PEG 6000) and an increase in the phage concentration from $0.87*10^3$ PFU/mL to $1.05*10^3$ PFU/mL was observed. The concentrated phages were found to be stable when stored at -20°C in PBS (Phosphate Buffer Saline) solution. The host range of isolated phages were tested by checking their lytic activity against beneficial pathogens such as *Pseudomonas fluorescens* and *Bacillus subtilis* found in tomato plants. This study forwards the idea of engaging bacteriophages as effective biocontrol agents rather than employing the harmful chemicals to treat bacterial speck of tomato plants.

***Key words*:** Tomato, *Pseudomonas syringae* pv. *tomato*, Bacterial speck, Bacteriophage, Bio-control agent

Microbiota play a key role in agricultural sector wherein they are either beneficial or pathogenic. Pathogenic microbes are primarily responsible for pre- and post-harvest losses amidst which bacterial pathogens predominate over fungal and viral diseases in agricultural crops. *Pseudomonas*, *Xanthomonas* and *Erwinia* sp. are the primary bacterial pathogens responsible for devastating losses in agricultural field. Most of the existing plant disease management strategies involve the application of chemicals in order to protect crops from microbial infections. But these are effective against several fungal pathogens and the excessive use of such fungicides has induced resistance in bacterial pathogens [1]. The Integrated Pest Management (IPM) strategy involve the application of chemical fungicides, pesticides, insecticides to curtail plant diseases and to increase food production. Application of such chemicals result in depleting the nutritional quality and the population of beneficial microbiota in the soil thus affecting the quality of the cultivable land which are also toxic to humans and animals [2]. Moreover, there exists several bio-control methods to control the infestation of microbial pathogens wherein the beneficial microbiota is being applied in large quantities [3].

Pseudomonas sp. is known for its diverse beneficial effect over plant growth and plant disease management but a very few are pathogenic. One among them is *Pseudomonas syringae* which is responsible for plant diseases such as bacterial cankers, stem and leaf spot, bacterial blight, speck, soft rot and galls [4]. A number of economically important diseases like halo blight of *Phaseolus vulgaris* [5], flower blast, necrotic leaf spots, shoot tip dieback, stem canker (gummosis), spots, specks and blisters on fruits are caused by this pathogen [6]. This bacterium infects a wide variety of fruits, vegetables, and ornamental plants. Symptoms associated with *Pseudomonas syringae* include flower blast, appearance of spots and blisters on fruit and development of stem cankers [7]. *Pseudomonas syringae pv. tomato* serves as the causal agent of bacterial speck on tomato plants which is observed as spots on leaves and fruits. The outbreak of bacterial speck was recorded in the late 1970s that prompted researchers to perform several ecological and epidemiological studies on bacterial speck pathogen [8-10].

Pseudomonas syringae pv. tomato finds its route through natural openings called stomata in leaves and lenticels in woody tissues. The bacteria produce toxic chemicals such as coronatine that keeps its stomata open, modifies host cells and

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thereby favours bacterial infestation [11]. Initially, small dark brown spots appear on the leaves by which it is difficult to discriminate between bacterial spot and bacterial speck. Later symptoms like burnt leaf margins resulting in stunted growth leading to the death of the seedling are the confirmative symptoms [12]. Reduction of yield is observed among tomato plants infected by *Pseudomonas syringae* pv. *tomato*. It is reported that the bacterial spot disease is the major infection that would cause nearly 30% loss in the crop yield. This disease mainly caused by pathogenic *X. vesicatoria, X. euvesicatoria, X. gardneri* and *Xanthomonas perforans* [13]. Removal of plant debris is highly recommended to prevent further infection of other healthy plants. Chemical control was done using copper compounds like the 'Bordeaux mixture', copper salts of fatty acids, ammoniacal copper, copper sulfate, cupric hydroxide and many other heavy metals. It was also carried by combining a fungicide or a pest control chemical. Antibiotics like streptomycin [14] or tetracycline alone or in combination with the above-mentioned copper compounds has produced varied results [15]. Application of certain insecticide like deltamethrin resulted in inducing resistance in *P. syringae* pv. *syringae* strains [16].

Bacteriophages are bacterio-specific viruses that exclusively target bacterial cells and its application is widely studied in recent years. They can be effectively applied as biocontrol agents against bacterial pathogens in agricultural sector that have acquired resistance to antimicrobial agents [17-18]. This method is reported to be effective over other physical, chemical and biological methods which being practiced to manage bacterial diseases in agricultural sector [19]. The present study describes the isolation of phages from *Pseudomonas syringae* pv. *tomato* infected tomato plants and to purify the isolated phages. The effect of phages over a few beneficial bacteria found in tomato plants is also reported.

MATERIALS AND METHODS

Infected leaves, stem and tomato were collected from an agricultural field in the southern part of Tamil Nadu, India during the month of January, 2016. The collected samples were stored at 4°C until further analysis.

Isolation of Pseudomonas syringae pv. tomato

The infected samples were cleaned and surface sterilized using ethanol (70%) and immersed in sodium hypochlorite solution for 3 minutes. About 1g of the treated sample was then immersed in sterile distilled water and serially diluted [20]. 1 mL from each dilution was plated on sterile Luria Bertani agar plates and incubated at 37°C for 48h. From the plates, based on the morphological appearance of the bacterial colonies, few repeating colonies were selected and cultured separately [21].

Biochemical tests

Biochemical tests were performed to screen for the presence of the bacterial speck pathogen, *Pseudomonas syringae* pv. *tomato*. Biochemical tests such as Gram staining, motility test, H_2S production test, citrate test, urease test, indole test, methyl red test, oxidase test and potato soft rot test were carried out by standard protocols mentioned in literatures to identify the targeted phytopathogen [22-25].

Isolation of bacteriophage

Phage enrichment from tomato leaves

The infected plant material that was collected was suspended in phosphate buffer for 60 min. The buffer solution provides a stable environment and protects the components in the collected suspension from extraneous factors. The liquid is collected and centrifuged for 5 min and filtered through 0.22µm syringe filter.

Double agar overlay test

Double agar overlay method was carried out to check for the occurrence of bacteriophage in the filtrate. Bacteria and phage lysates were mixed in different proportions and incubated at 37ºC for 5 min to facilitate effective adsorption. The incubated sample was added to 5 mL of LB soft agar (0.5%). The inoculated soft agar was transferred onto LB hard agar (1.5%). Formation of plaques could be observed after incubation at 37ºC for 24-48 h [26-27].

Turbidity test

Meanwhile, the phage lysate (1mL) was added to overnight grown bacterial culture and incubated at 37ºC for 24 h. The lytic activity of the bacteriophage was ascertained by the turbidity change in the medium [28].

Purification of phages

PEG 6000 was employed for precipitation of the isolated plaques [29]. The plaques were scraped out from the soft agar layer and suspended in distilled water and centrifuged at 10,000 rpm for 30 min at 4ºC. The supernatant was treated with 10% PEG at room temperature and subsequently kept in an ice bath for 1 h. The pellet obtained was resuspended in 5mL of saline (0.9% NaCl) [30-31].

Host range analysis

The host range of bacteriophages was determined by spot test method [32]. The sensitivity of bacterial isolates towards the phage isolates was determined. 5 mL of soft agar mixed with 100 µL of the bacterial culture and an equal volume of $CaCl_2$ (300mM) was overlaid onto hard agar. 3µL of phage lysate was dropped on soft agar and the plates were incubated at 37ºC for 48 h. The lytic ability of phages was confirmed by the appearance of plaques. SM buffer spotted on the soft agar was kept as control [33-34].

Stability analysis

Stability of the isolated phages is an important constrain during storage. Therefore, isolated plaques were resuspended in PBS and stored at -20°C. Samples were analyzed at regular intervals by agar overlay method to detect the reduction in titer, if any.

RESULTS AND DISCUSSION

Isolation of Pseudomonas syringae

Bacterial isolates were obtained from the infected leaves of the tomato plant and were subjected to serial dilution. Six well defined colonies from the serially diluted plates were selected and streaked onto LB agar plates and the colony morphology was observed. Colonies which were round and creamy in nature were subjected to biochemical tests [35]. As mentioned in Shila *et al*. [36], the colony morphology of *Pseudomonas syringae* pv. *tomato* is round and creamy. Such kind of colonies from the initial culture plates were identified and cultured further. (Table 1) shows the biochemical test results observed for the bacterial isolates.

Isolation of bacteriophage

Agar overlay method was followed to obtain clearance zone on the bacterial lawn. Different dilutions of *Pseudomonas*

syringae pv. *tomato* and the phage filtrate were added together to enhance phage adsorption on to the bacterial host. Each such phage-bacterial mixture resulted in the formation of plaques in agar plates and the plates with highest number of plaques were considered as the optimized ratio which is 1:5 (Bacteria: phage). Appearance of clear plaques confirms the lytic ability of bacteriophage population in the filtrate and the concentration of phages was found to be $0.87*10^3$ PFU/mL.

Table 1 Biochemical test results

Tests	Bacterial isolates					
	1	2	3	4	5	6
Gram staining	-	-	-	-	-	-
Motility	+	-	+	-	+	+
H_2S production test	+	+	+	+	+	+
Citrate test	+	+	+	+	+	+
Urease test	+	+	-	-	+	+
Indole test	-	-	-	-	-	-
Methyl red test	-	+	-	-	-	+
Oxidase test	+	+	+	+	+	-
Potato soft rot test	-	-	-	-	-	-

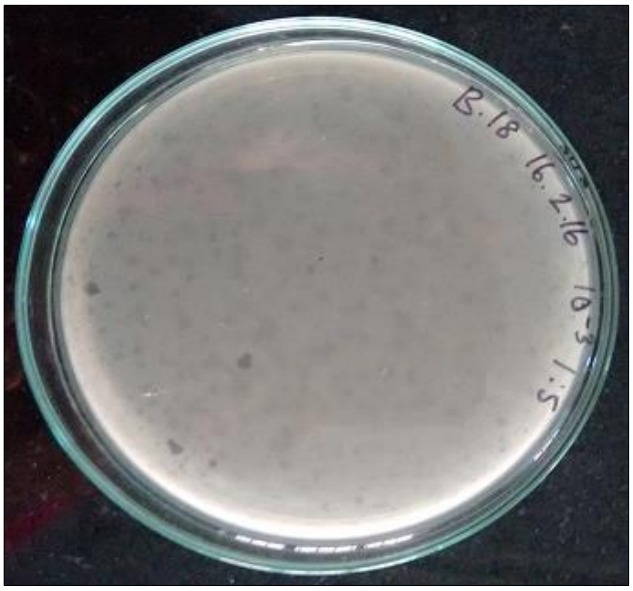

Fig 1 Purified phages subjected to double layer agar overlay method

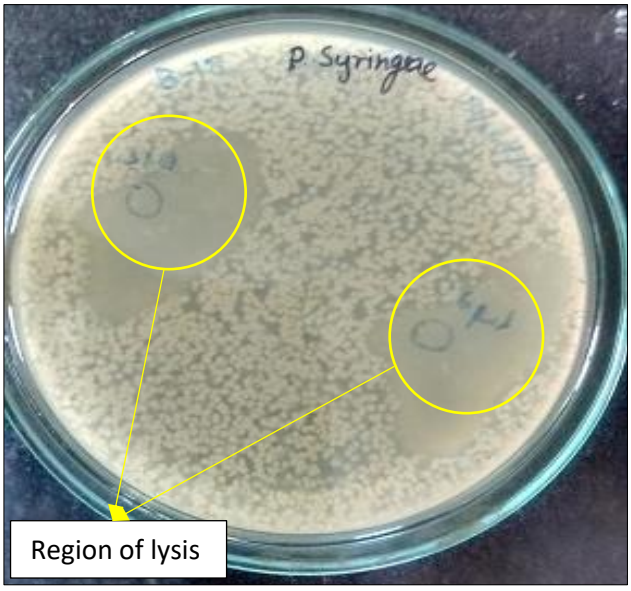

Fig 2 Test for lytic activity of the phage on *Pseudomonas syringae* pv. *tomato*

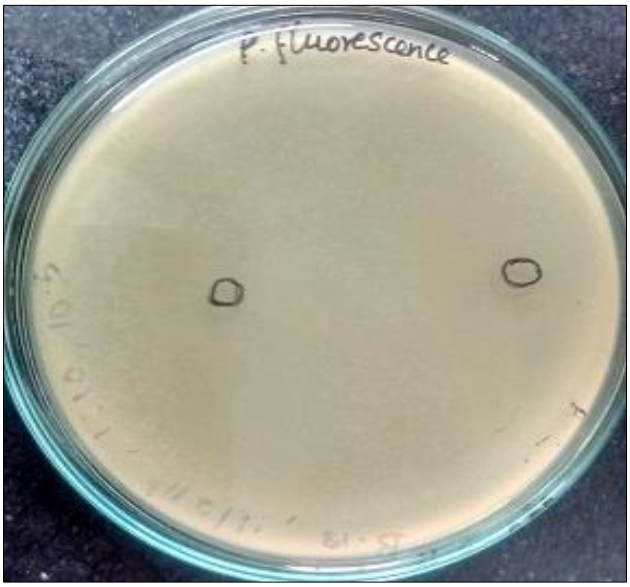

Fig 3 Test for lytic activity of the phage on *Pseudomonas fluorescence*

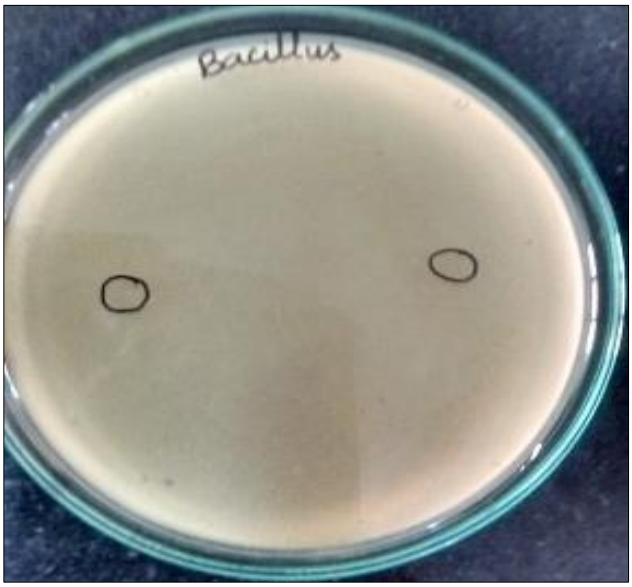

Fig 4 Test for lytic activity of the phage on *Bacillus subtilis*

Turbidity analysis: Change in turbidity of the culture media was observed after 24h which reveals the lytic activity of the isolated bacteriophages. The change in turbidity of the medium is due to the lytic activity of phages which decreased

the optical density of the culture media. This reveals that the isolated phages are specific to the targeted bacterial species [37].

Concentration of phages

The PEG purified phages were plated by double layer agar overlay method (Fig 1). An increase in the concentration of phages upto $1.05*10^3$ PFU/mL was observed. PEG 6000 helps in the recovery of intact phages and it is involved in protein precipitation. The bacteriophages are made up of proteins that encapsulate the genetic material [38]. Therefore, employing PEG 6000 enhanced phage precipitation which furthermore increased the concentration of phages.

Host range analysis

The activity of *Pseudomonas syringe* pv. *tomato* phage against different bacterial strains was tested. *Pseudomonas fluorescence* and *Bacillus subtilis* are the two major beneficial bacteria found in tomato plants. They were included in the host range analysis to ascertain the specificity of phages towards lysing *Pseudomonas syringae* pv. *tomato* [39-40].

(Fig 2) reveals the lytic activity of phages against *Pseudomonas syringae* pv. *tomato.* Absence of zone of clearance in (Fig 3-4) confirms the inability of the phages to lyse *Pseudomonas fluorescence* and *Bacillus subtilis*. The isolated phages are found to be species specific since they show no lytic activity towards *P. fluorescence* species belonging to the same genera. These findings suggest that the isolated phages possess narrow host range which is an important feature of any biocontrol agent and that the harmful bacteria alone are targeted.

Stability analysis

No reduction in phage titer during the entire study period was observed when the phages were stored in PPB buffer at -20°C. Not all phages penetrate bacterial cells at temperatures other than the optimum levels and therefore the multiplication of phages gets affected [41]. This might be the cause of considerable reduction of phage titer at unfavourable temperature conditions. It has been reported that the thermal stability of the viruses is influenced by the cholesterol level in the viral lipid shell and host cellular plasma membrane [42]. This could also be a source of instability.

CONCLUSION

Bacteriophages effective against *Pseudomonas syringae pv. tomato,* the causative agent of bacterial speck in tomato plants were successfully isolated and purified using PEG 6000. The specificity of isolated phages towards *Pseudomonas syringae* pv. *tomato* proved its target specific activity. From this study, it is suggested that the isolated *Pseudomonas syringae* pv. *tomato* specific phages could be used to treat bacterial speck of tomato crop plants. The feasibility of large-scale production and stability enhancement of phages must be studied to confirm its production in industries. With an effective and stable phage biocontrol formulation for field application, phage therapy will benefit farmers in an emerging scenario of bacterial resistance against antimicrobial agents.

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