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Isolation of lytic bacteriophage against *Ralstonia solanacearum* causing wilting symptoms in ginger (*Zingiber officinale*) and potato (*Solanum tuberosum*) plants

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Abstract

This research work is aimed to find lytic activity of bacteriophage against phytopathogenic bacteria. Ralstonia solanacearum is a soil inhabiting bacterium colonizes in roots and tubers in various plants results in wilting in many vegetable crops especially in solanaceous plants. Ralstonia solanacearum invades in roots, stem and xylem vessels within the host and extensively multiplies in potato and ginger crops leads to economical losses. The bacteria were isolated from infected potato and ginger tubers. The isolates were characterized by TTC media and PCR based detection with Ralstonia solanacearum specific primers (F: 5'-GTCGCCGTCAACTCACTTTCC-3', R: 5'-GTCGCCGTCAGCAATGCGGAATCG-3'). Use of bacteriophages for the control of plant bacterial diseases is fast expanding methodology over chemical aided control of bacteria. Therefore, we made attempt to isolate a lytic bacteriophage against Ralstonia solanacearum. The efficacy of phages as antimicrobial agents for specific bacteria is a relavant technology to overcome bacterial diseases without affecting the beneficial micro-flora of the soil. We could able to demonstrate the viable bacteriophages acts as omnilytics against specific bacteria. Bacteriophage ϕHMPM-2012 was isolated against the phytopathogenic Ralstonia bacteria by soft agar diffusion method. In vitro confirmation of bacteriophage activity and electron microscopic studies were done. We are proposing phages as effective biocontrol agents in plant protection

Keywords: *Ralstonia solanacearum,* phytopathogen, bacteriophage, bio-control agent.

Introduction

Ralstonia solanacearum was originally described by Smith (1986) and previously known as *Pseudomonas solanacearum* as the causative agent for wilting disease in many plants species including potato, tomato, tobacco, banana, peanut and ginger. It is resulted in substantial yield lose in worldwide[1,2]. On the other hand this is a soil bacterium and causal agent of devasting wilting disease of major on *solanaceous* vegetable crops. As it resulted economic and social impact from its wide distribution in all the tropical countries of the world. *Ralstonias olanacearum* (synonym *R. solanacearum* E. F.Smith)[3] has been resulted in widespread bacterial wilting disease of most important crops in the globe.

This phytopathogenic bacterium has been recorded on hundreds of species representing 44 botanical families of plants[4]. *R. solanacearum* enters through the wounds of the plant root from the handling equipment of cultivation, nematodes, insects and through cracks of secondary roots[5]. Bacterial wilt is one the important disease in many crops is caused by *Ralstonia solanacearum* and its subgroup, formerly called *Pseudomonas solanacearum.* The strains of *R. solanacearum* were classified into five races and five biovars based on there on host range and their ability to produce acid from a panel of carbohydrates[6]. *Ralstonia solanacearum* is a soil borne phytopathogenic β-proteo-bacterium and exhibited wide host range distribution. 54 botanical families were affected in tropical and subtropical midland countries of the world. Recently, colonization of *Ralstonia solanacearum* and wilting symptom was observed in tomato plant[2,7,8]. Bacteriophages are the kingdom of viruses that infect the specific bacteria, and are distinct from the animal and plant viruses. Phages can kill the bacteria either a "lytic" or "lysogenic" mode of life cycle. The lytic bacteriophages are the most suitable natural enemies of bacteria has been used in phage therapy because they show quick infection to the specific host range, growing exponentially in number and results in killing of bacteria[9]. The complete genome analysis of bacteriophage specific for *Erwinia amylovora* a causative agent fire blight, a plant disease was extensively been studied. The study provided the complete annotations of the complete genome of *E. amylovora* specific bacteriophages[10]. Recently one of the study anticipated the use of alternative bacteriophage mediated control of bacteria. The use of three *Ralstonia* specific phages ϕ RSA1, ϕ RSB1 and ϕ RSL1 has been reported to be useful for the eradication of wilt causing bacteria[11].

A research on DNA isolation and PCR based identification of bacterial pathogen was greatly anticipated the use of universal *Ralstonia solanacearum* specific primer. The most reliable PCR based detection of phytopathogenic bacteria was employed[12]. The research of phages based on *Salmonella* were isolated from sewage effluent and the use of phage treatment for the reduction of *Salmonella* was extensively studied[13]. The phage research and utilization of filamentous bacteriophage for the control of *R. solanacearum* has led to new trend in phage mediated control of phytopathogenic bacteria. Loss of virulence of phytopathogenic *R. solanacearum* through filamentous phage treatment has been extensively studied and the study suggested that, phages may be the effective tools for the control of bacterial pathogenicity in plants[14,15]. The investigation on lytic nature of bacteriophage for the control on the wide range of *Ralstonia* has shown the efficient use of phages to kill the pathogenic bacteria of tomato plant[16]. However, we think that there is a arena of literature and sufficient data on bacteriophage studies may show promise for treating phytopathogenic bacteria. To facilitate further progress in phytopathology, directions for future research, we are proposing to find out new alternative bio-control modalities in the field of phage research for plant protection.

Material and Methods

Collection of samples and bacterial isolates: The bacterium *Ralstonia solanacearum* was isolated from wilt infected potato and ginger plants collected from Chikmagalur and Hassan districts, Karnataka, India from 2010 to 2011 (figure 1(a) and 1(b)) 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. Bacterial ooze from plant samplewas 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[17, 18]. 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.

Pathogenicity test: Seven isolates of *Ralstonia solanacearum* were grown on TTC containing petri plates for 48 h at 28°C. The white pinkish irregular colonies were harvested and suspended in sterile distilled water. The bacterial suspension was further diluted up to desired concentration of bacteria ($1x10^9$ CFU/mL). Tomato and chilli was grown in pots at 27°C for pathogenicity test. Three plants were maintained in each pot and replicated thrice. The plants were inoculated at root zone by making slight injury to the root with disposable syringe and 5 mL of inoculum was poured to injured portion. The inoculated plants were observed regularly for wilting symptoms[17].

Figure-1(a)
Infected potato plant and tuber

Figure-1(b)
Infected ginger plant and tuber

Bacterial DNA extraction: *R. solanacearum* strains were grown for 2 days in liquid media supplemented with yeast-petone-glucose (YPG) agar medium containing following constituents (per litre): yeast extract 5g, Peptone 5g, glucose 5gand agar 5g. Bacterial culture was incubated at 28°C. For DNA extraction, 50 ml of cell culture were pelleted by centrifugation for 10 minutes at 1000X 3g[19]. Thereafter, collected bacterial cells were washed once in sterilized distilled water and subjected to lysis byadding sodium dodecyl sulphate (SDS) to a final concentration of 1% (w/v). Subsequently, DNA was isolated and purified by standard phenol-chloroform[20].

PCR based detection of *R. solanacearum* using Rs specific primers: PCR based detection of *R. solanacearum* using Rs specific primer was done. PCR amplification for detection of mixture (25 µL) with PCR buffer $MgCl_2$:1.5mM, dNTP mix: 0.05mM, DNA polymerase enzyme: 0.5U, Bacterial template DNA: 100mg, BSA: 10mg, (PCR Kit, Aristogene Biosciences, Ltd, Bangalore, India), Primers: 20 pmoles each (Forward primer: 5'-GTCGCCGTCAACTCACTTTCC-3'; Reverse primer: 5'-GTCGCCGTCAGCAATGCGGAATCG-3'). PCR amplification of DNA was performed in Effensdorf gradient thermal cycler at the suitable conditions for PCR[12,21] and the PCR amplified products were separated in 1% agarose gel with 0.6 µg/mL of ethidium bromide for 1h at 60V constant voltage.

Electrophoresed gel was photographed for the detection of DNA bands on a UV transilluminator. The results were documented in Alpha imager Gel Doc system.

Isolation of *R. solanacearum* phage: The isolation of bacteriophage was done according to the method of Smith and Huggins. The bacteriophage was isolated from raw water of municipal sewage treatment plant, Hassan, Karnataka. 50 mL of raw sewage water was collected with few drops of chloroform in a sterile conical flask. Equal volume of lactic phage broth was added and 1ml broth of 24 h old culture of *R. solanacearum* was incubated at 37ºC for 24 h. After 24 h of incubation, lysate was added with few drops of chloroform and shaken for about 15 minutes. The mixture was centrifuged at at 10000 rpm for 15 min, supernatant was filtered through 0.22 µm pore size Acrodisc membrane filters (Pall, German Laboratory) to remove the lysate of bacteria[23]. 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[12]. The plaque forming (PFU) assay was conducted using double-layer agar diffusion method previously described by Smith and Huggins[12]. 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 isolation of single phage type[24]. The phage isolated was designated as *Ralstonia solanacearum* phage ϕHMPM-12.

In-vitro confirmation of phage lytic activity against *R. solanacearum* and host range specificity: 1.0 mL of 24 h old culture of *R. solanacearum* bacterial lawn was flooded on nutrient agar petri palates, excess of culture drained out and wells were dug into agar with the help of sterile cork borer and 25 µL of phage ϕHMPM-12 suspensions ($3X10^9$ PFU/mL) was loaded in to each of the well. Sterile distilled water was used as negative control. All the experiments were conducted in quadra-duplicates and petri plates were incubated at 37ºC for 24 h for phage lytic activity. The zone inhibition was recorded. The host range susceptibility of isolated phage ϕHMPM-12 was also determined with other pathogenic bacteria including *Staphylococcus aureus*, *Staphylococci* and *Pseudomonas aeruginosa* to evaluate genus specificity[2,7].

Study of bacteriophage morphology by transmission electron microscope: Bacteriophage ϕHMPM-12 solution was 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 as described previously[8,25] and morphology of phages were observed in transmission electron microscope. Morphological images of isolated phages were recorded.

Results and Discussion

Isolation of bacteria and use of specific media: Fluidal colonies with characteristic pink centered appeared after 48 h CPG medium. The different colony morphology was observed that the colonies were irregular, white and fluidal with incubation period of 48 to 72 h on CPG and SMSA media. Both bacterial isolates from ginger and potato exhibited similar characteristic pink center and it was also observed that, the white colonies were observed when bacterial isolates grown on nutrient agar media. Fluidal pinkish red centered colonies of typical *R. solanacerum* were observed on TTC media (figure 2a). The pink colored colonies were isolated and purified for confirmation of bacterial wilt causing pathogenicity of *R. solanacearum*. Virulent colonies appear white with pink colour at the centre. It was also observed that white colonies were developed on nutrient agar media (figure 2b).

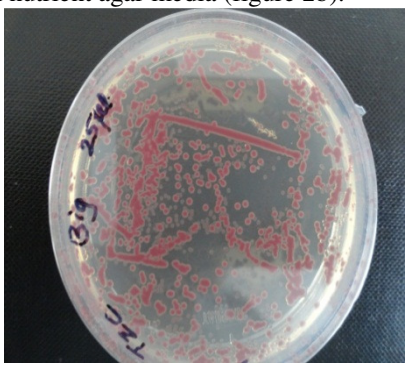

Figure-2(a)
***Ralstonia solanacearum* colonies on TTC media**

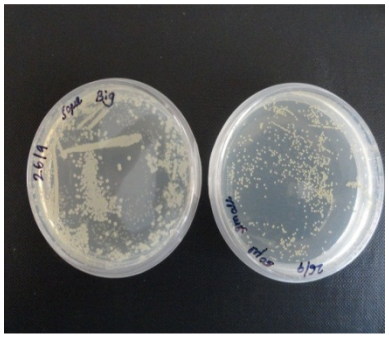

Figure-2(b)
colonies of *Ralstonia solanacearum* on nutrient agar media

Pathogenicity of bacterial isolates: It was shown that all the isolates from potato and ginger induced wilt symptoms in tomato and chilli plants. Interestingly *R. solanacearum* isolates exhibited wilting symptoms 3 to 4 days after inoculation and all the inoculated plants wilted within 5 to 10 days (figure-3). Earlier[26], research has reported quick pathogenicity induced wilting of tomato and chilli plants, but the present research strongly identifies bacterial isolates of both ginger and potato plants were induced quick wilting symptoms after 3 days of inoculation.

Figure-3
***Ralstonia solanacearum* strains induced pathogenicity wilting symptoms on chilli and tomato plants**

PCR based detection of *R. solanacearum* using Rs specific primers: The sensitivity of the present study was greatly anticipated use of Rs specific primers for the amplification of target genomic DNA of *R. solanacearum* isolated from infected potato and ginger plants. The sensitivity of PCR based method has remarkably found that Rs primers amplified all the strains of bacteria. This study was greatly attributed the detection of phytopathogenic bacteria by means of PCR technique. Previous studies suggested use of PCR for detection of pathogenic bacteria. The recent research on PCR based method using Rs specific primers has been developed for *R. solanacerum* has been mentioned in known to amplify 281bp in the genomic DNA[12]. Primer sequence[21] has been known to amplify 281 bp sequences in the genomic DNA of *R. solanacearum.* The intensity of amplified product using Rs specific primer amplified 280 bp in bacterial genome of all isolated bacterial samples of infected potato and ginger plants (figure-4). The sensitivity can be further improved by enrichment of soil suspension in selective medium for *R. solanacearum* as selective medium has already been developed for this bacterium[27]. In conclusion, this research strongly recommends use of PCR based detection of phytopathogenic *R. solanacerum.*

Isolation of phage, host range activity, electron microscopic studies: Phage plaques were formed after 24 to 48 h of inoculation with *R. solanacerum* bacteria, 25-60 plaques are resulted per Petri plates (figure-5). Formation of the plaques indicated antimicrobial against specific bacteria. These phages may be useful has an antimicrobial agent for the eradication of phytopathogenic *R. solanacerum* strains. Based on the infectivity and morphological studies on bacteriophages can be characterized into specific modality. Isolated phage ϕHMPM-12 was exhibited to form plaques on 60 % of the *R. solanacerum* and effectively inhibited the growth the bacterial isolates. Thus isolated bacteriophage proven its antimicrobial activity against the phytopathogenic *R. solanacerum.* The susceptibility of phage ϕHMPM-12 was shown narrow range activity on isolated bacterial strains of *R. solanacerum*. Phage ϕHMPM-12 was shown negative antimicrobial activity against *Staphylococcus aureus*, *Staphylococci* and *Pseudomonas aeruginosa,* thus phage ϕHMPM-12 has shown genus specificity. Further bacteriophages were propagated and purified from single plaques isolated and electronic microscopic images of the phages revealed that bacteriophages have typical icosahedral head about 65 nm in diameter 100 nm tail part (figure 6.) as previously described coliphage P2 like morphology[28, 29]. Based on the morphological relevance of isolated phage ϕHMPM-12 resembles to siphoviridae family. In conclusion *Ralstonia* is a genus of proteo–bacteria it is soil borne and motile bacteria. It colonizes in xylem which finally resulted in wilting of plants most of the *Solanaceous* members are infected by this bacterium. Due to this devasting lethality, we have isolated bacterium from infected plants of potato and ginger fields. Further we characterized the isolated bacteria as a member of *Ralstonia* by use of specific media (TTC) and PCR based detection of *R. solanacerum* using Rs specific primers. The Bacteriophage was isolated from sewage water and filtered through Acrodisc filter and later it was introduced into plate in which bacteria were cultured and formation of plaques were observed. Further the attack of bacteriophages on bacterium was confirmed by transmission electron microscope.

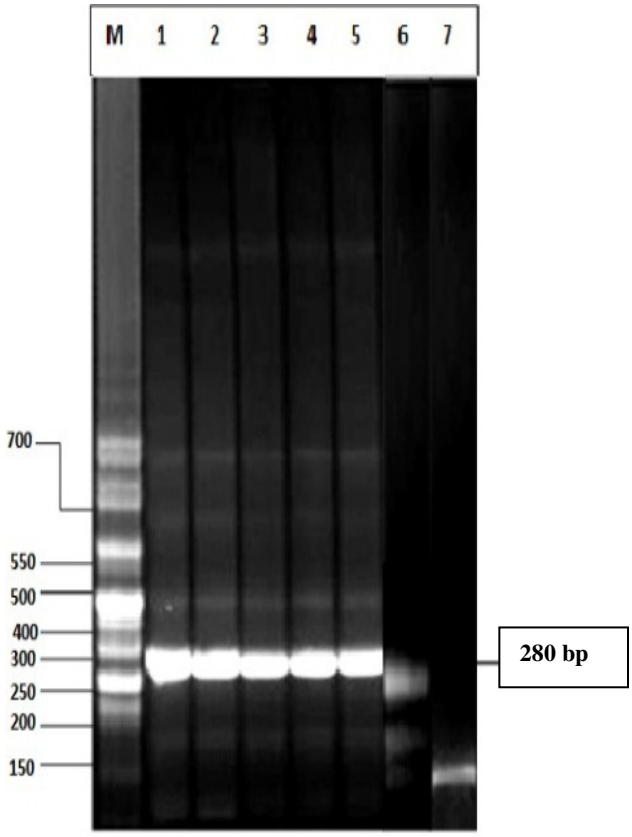

Figure-4
PCR based detection of Phytopathogenic *R. solanacearum* using Rs specific primers M: DNA size marker (bp) Phi X 174 Hinf digest, Lane 1-4: DNA extracted from bacterial isolates from infected potato and ginger plan

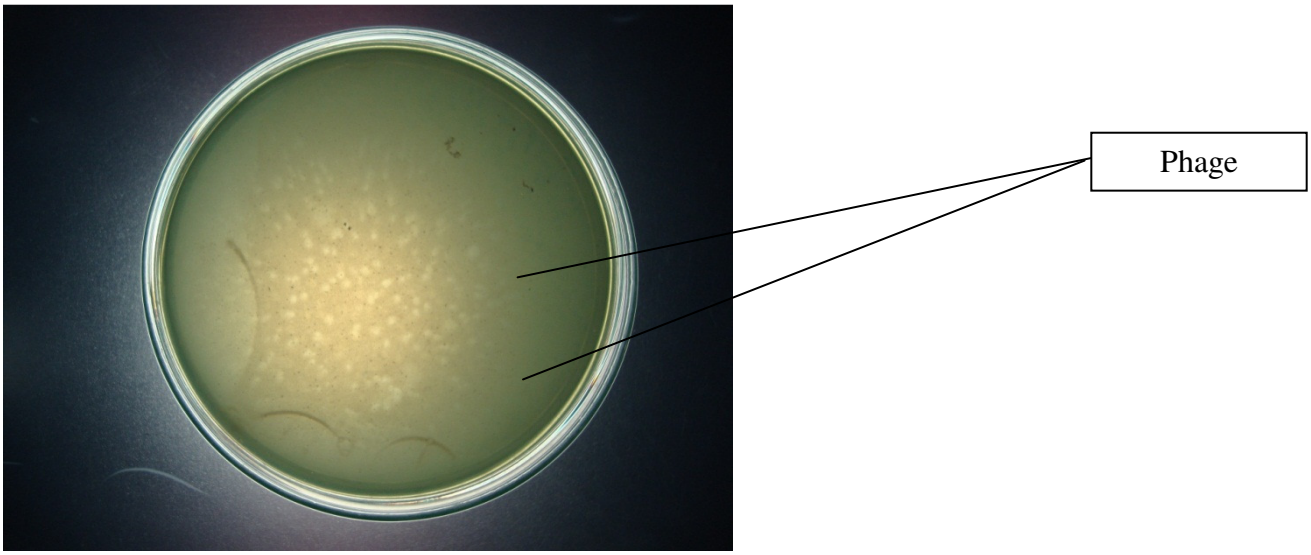

Figure-5
Formation of plaques on *Ralstonia solanacearum* culture plate

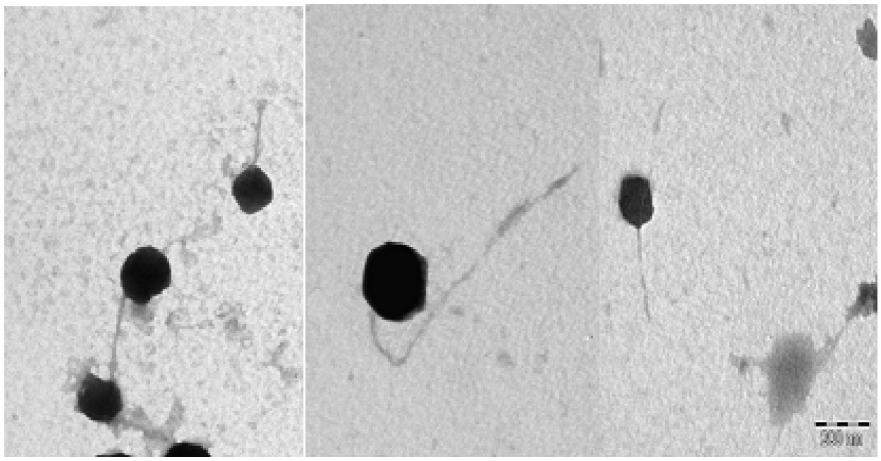

Figure-6
Transmission electron micrographs showing morphology of bacteriophages infecting *Ralstonia solanacearum.* Head and tail structures are seen for phage ϕHMPM-12

Conclusion

The findings of this research greatly anticipated the use of phages as antimicrobial agents to *R. solanacerum* strains. Detection of pathogenic *R. solanacerum* through PCR based Rs specific primers is a most reliable methodology for fast identification of specific bacteria. Phages ϕHMPM-12are potent omnilytic and lethal to *Ralstonia* species. Thus it can be used as bio-control agent *in vitro*. A further application of this technique *in vivo* is to be done essentially. Thus we are proposing new modalities of bacterio phages for the control of phytopathogenic bacterial infections in plants.

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