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Bacteriophages: A New Weapon for the Control of Bacterial Blight Disease in Rice Caused by *Xanthomonas oryzae*

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Xanthomonas oryzae, **a bacterial pathogen causing leaf blight disease (BLB) in rice, can cause widespread disease and has caused epidemics globally, resulting in severe crop losses of 50% in Asia. The pathogen is seed-borne and is transmitted through seeds. Thus, control of BLB requires the elimination of the pathogen from seeds. Concern about environment-friendly organic production has spurred improvements in a variety of biological disease control methods, including the use of bacteriophages, against bacterial plant pathogens. The present study explored the potential of bacteriophages isolated from diseased plant leaves and soil samples in killing the bacterial pathogen in rice seeds. Eight different phages were isolated and evaluated for their bacteriolytic activity against different pathogenic *X. oryzae* strains. Of these, a phage designated φXOF4 killed all the pathogenic *X. oryzae* strains and showed the broadest host range. Transmission electron microscopy of φXOF4 revealed it to be a tailed phage with an icosahedral head. The virus was assigned to the family *Siphoviridae*, order *Caudovirales*. Seedlings raised from the seeds treated with 1×10^8 pfu/ml of φXOF4 phage displayed reduced incidence of BLB disease and complete bacterial growth inhibition. The findings indicate the potential of the φXOF4 phage as a potential biological control agent against BLB disease in rice.**

Keywords: ***Xanthomonas oryzae,*** **bacterial leaf blight disease, bacteriophage, biological control**

Introduction

Xanthomonas oryzae pv. o*ryzae* [1], the causal agent of bacterial leaf blight (BLB) disease in rice, continues to be the most devastating disease in rice which has resulted in up to 50% yield losses [2]. The leaf blight disease is one of the earliest known diseases and was reportedly noticed by farmers in Japan during 1884 [3]. It has been reported that the remains of infected plant, irrigation water and weed hosts or seeds served as source of the pathogen [4, 50]. The leaf blight disease of rice has been reported to be seed borne in nature [6]. It has been observed that the presence of *X. oryzae* on rice seeds have not produced any perceptible symptoms [5, 7, 8]. The pathogen has also been found occasionally in glumes and endosperm of seeds harvested from heavily infected rice fields [9]. Perhaps, the isolation of the pathogen from seeds of infected plants [7, 10, 11] and detection of its presence in rice seeds by molecular techniques has become a major concern worldwide as large quantities of seeds are frequently transported from one country to another for cultivation purposes and for breeding programs. This might have contributed to the spread of the pathogen to new cultivars in a particular

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area which once were pathogen free.

As part of a conventional disease management strategy, efforts have been put forth for disinfection of seeds to eradicate seed borne pathogen. The process of rice cultivation includes germination of the seeds in nursery followed by transplantation in the field. It has been observed that the yield loss has been found to be higher when the plants are infected by the pathogen at the nursery stage [9]. Effective control measures are required to disinfect the seeds to avoid the shortage of seeds and seedlings for plantation. Gnanamanickam *et al.*, have recommended three major management strategies for disinfection of rice seeds such as (a) application of chemical pesticides, (b) biological control and (c) use of resistant host plants [12]. The contemporary control measures include sterilization of seeds with chemical pesticides dissolved in aqueous solvent, which generates pesticide residues that have potential for contamination of the ground water. Another control measure is treatment with hot water [14] which has been reportedly affects the germination percentage of the seeds. Therefore, in the recent past, emphasis has been put forth for the sustainable agriculture with eco-friendly approaches and evasion of chemical pesticides which have warranted exploration of newer control methods.

One such eco-friendly approach is employing bacteriophages as bacterial disease control agents [15, 16]. Bacteriophages are viruses that infect bacteria and they have peculiar and desirable properties their omni presence, specificity and exponential propagation which make them as an excellent biocontrol agent. The Environmental Protection Agency, USA (http://www.omnilytics.com/products/agriphage) has approved a pesticide containing bacteriophage (AgriPhage™) for biological control of bacterial diseases, especially for the control of bacterial spot and bacterial speck of tomato and pepper plants. Several studies have been made earlier [17–21] to utilize phages as biocontrol agent against plant bacterial pathogens however only limited number of investigation has been done for disinfection of seeds with phages. Disinfection of seeds with bacteriophages against seed borne bacterial pathogen has been successfully carried out [22, 23]. It has been envisaged that the major advantage of sterilizing the seed with phages is that the phages make more contact with pathogen which leads to more killing of the pathogen. In addition, bacteriophages have been used to detect BLB disease symptoms in the field [9, 24–26], however there is no report on the usage of phages to disinfect BLB contaminated rice seeds. Therefore, an attempt has been made in the present study to evaluate the potential of bacteriophages as biological control agents against *X. oryzae* and to develop it as an effective biopesticide for the control of bacterial blight disease in rice.

Material and Methods

Sample collection

The infected rice plant samples were collected from the research fields of Acharya N.G. Ranga Agricultural University (ANGRAU), Maruteru, Andhra Pradesh which has been declared as the hotspot for BLB in the coastal Andhra Pradesh [27]. Infected leaf, soil and water samples were also collected from the farmers' fields adjoining the research station. The sampling was done in the form of a "W", wherein each terminal point was considered as a hill and this was done to cover the entire infected field. Two infected leaves were collected per hill. A total number of 45 infected leaf samples were collected from the fields and used for the study.

Isolation and identification of *X. oryzae*

The diseased leaf samples were brought to the laboratory and the bacterial pathogen was isolated according protocol prescribed by Bradbury [28] and characterized. Briefly, the samples were surface sterilized with sodium hypochlorite (0.1%; w/v) for 2 min, the infected portions of the leaf were cut into small pieces from the advancing margin of the lesions with a sterile scalpel and incubated in vials containing 2 ml sterile deionized water for 5–10 min. A loop was inserted into the water containing the bacterial ooze and the bacteria were streaked on Peptone Sucrose Agar (PSA) medium (sucrose 10 g, peptone 10 g, N-sodium glutamate 1 g, agar 18 g Hi Media Biosciences, Mumbai). The plates were incubated at 28 ± 2℃ for 4–5 days [10] and examined for the appearance of pale yellow, pin head sized and mucoid bacterial colonies. The pathogenicity of the isolated strains of bacteria was verified by infecting them onto the seedlings of susceptible rice cultivar, Taichung Native (TN1), a gift from ANGRAU, Andhra Pradesh. Bacterial inoculum was prepared by suspending 48h

Table 1. Details of *X. oryzae* isolates collected from bacterial blight infected plants.

S. No.	Sample collection sites	Cultivar from which isolated	Laboratory name given to the isolate
1	RARS Maruteru, Experimental Field, West Godavari District, Andhra Pradesh	MTU 1064	PPXAP1
2	RARS Maruteru, Experimental Field, West Godavari District, Andhra Pradesh	MTU 1064	PPXAP2
3	Maruteru , West Godavari District, Andhra Pradesh	TN1	PPXAP3
4	Maruteru, West Godavari District, Andhra Pradesh	TN1	PPXAP4
5	Maruteru, Krishna District, Andhra Pradesh	MTU 6024	PPXAP5

grown bacterial culture in 10 ml of sterile distilled water to a density of 1.2×10^9 cfu/ml. The inoculation was done on three fully expanded leaves of the 30 day old rice seedlings by leaf-clip method. The appearance of symptoms was observed after 10–15 days after inoculation.

Molecular identification of *X. oryzae*: The molecular identification of the bacteria was performed by amplification of 16S rRNA gene from the isolated bacteria. The genomic DNA from the isolated bacteria was extracted with the method developed by Gabriel and De Feyter [29]. The isolated DNA was used as the template for polymerase chain reaction (PCR) performed with *X. oryzae* specific forward XOR-F5'-GCA TGA CGT CAT CGT CCT GT3' and reverse XOR-R5'-CTC GGA GCT ATA TGC CGT GC-3' primers [15, 30]. The PCR reaction was carried out in 10 µl reaction volume which contained, 5 µl Master mix containing dNTPs and Taq DNA polymerase (Ampliqon Taq DNA Polymerase Master Mix RED), 0.5 µl each of forward and reverse primers at 10 picomole concentration and 1.0 µl template DNA (100 ng/µl). The amplification was performed with the following programme, initial denaturation at 94℃ for 10 min, denaturation at 94℃ for 30 sec, primer annealing at 60℃ for 30 sec, primer extension at 72℃ for 60 sec, and final extention at 72℃ for 10 min for 30 cycles. The amplified product DNA (10 µl) was analysed on agarose gel (1% w/v) as described by Sambrook *et al.* [31].

Isolation of bacteriophage

Bacteriophages were isolated from bacteria collected from soil and water samples by following the method of Crosse and Hingorani [32]. Approximately 10 g of soil was taken in a sterile 50 ml centrifuge tube, filled with the sterile tap water to the rim of the tube and allowed to stand for 20 min with periodic inversions. The tubes were then centrifuged at 6500 ×*g* for 20 min and the supernatants were filtered through 0.2 µm syringe filters. The filtrates were either used to detect the presence of phages immediately or stored at 4℃ for further use. In the present study the peptone sucrose agar medium was used as the basal medium for growing bacterial lawn and isolation of bacteriophages. The standard double agar technique proposed by Adams [33] was used to isolate phages. A mixture containing 0.2 ml of indicator bacteria, *X. oryzae,* and 0.1 ml of diluted sample containing phage was added to 3 ml of molten PS top agar (0.6%) medium. The mixture was poured on top of the bottom agar (1.5%) and incubated at 28℃ overnight. The phages were identified as plaques in a bacterial lawn and different phages were distinguished based on differences in plaque morphology. Individual phages were collected in vials containing SM buffer (NaCl 5.8 g, $MgSO_4 \cdot 7H_2O$ 2 g, 1 M Tris HCL (pH 7.4) 50 ml and 2% gelatin 5 ml per liter) and stored at 4℃.

Determination of host range specificities of isolated phages

The bacteriolytic potential of each of the isolated bacteriophage was determined by phage typing against 5 different isolates of *X. oryzae* presented in Table 2. All the *X. oryzae* cultures were streaked on to double agar plates as a thick line and once the streak has dried, 5 µl phage suspension was spot inoculated in the middle of the bacterial streak. The spots were allowed to dry and plates were incubated for 1–2 days at 28℃.

Determination of Bacteriophage Insensitive Mutants (BIM)

The frequency of emergence of bacteriophage insensitive mutant was determined as per the protocol described by Adams [33, 34]. The phage lysate was filtered through 0.22 µm syringe filters and the filtrate

Table 2. Sensitivity, plaque size measurement and host specificity of isolated phages.

Name of phages	Sensitivity to chloroform	Diameter of plaque (mm)	Bacterial hosts					
			PPXAP1	PPXAP2	PPXAP3	PPXAP4	PPXAP5	SR PPXAP1
φXOF1	Insensitive	3	+	+	+	-	-	+
φXOF2	Insensitive	3	+	+	+	-	-	+
φXOF3	Insensitive	3	+	+	+	+		+
φXOF4	Insensitive	3-4	+	+	+	+	+	+
φXOT1	Insensitive	4	+	-	+	-	-	+
φXOT2	Insensitive	4	+	-	+	-	-	+
φXOM1	Insensitive	4	+	-	+	-	-	+
φXOM2	Insensitive	5	+	-	+	-	-	+

+: Postive, for phage lytic activity; -: Negative, for phage lytic activity.

was used for spot testing. Briefly, 100 μl of overnight *X. oryzae* culture (PPXAP1) was added to 4 ml of molten PSA medium containing 0.8% agar and mixed. The soft agar was poured immediately onto the bottom agar and allowed to solidify for 15 min. Ten microliters of phage lysate was spotted onto the soft agar seeded with bacterial culture. The plates were incubated for one week at room temperature (28℃) and examined for appearance of bacteria in the lysis zones. The presence of bacteria indicates the emergence of bacteriophage insensitive mutants.

Characterization of phage φXOF4

One step growth curve: One step growth curve analysis was used to characterize the phage life cycle as per the modified procedure of Ellis and Delbruck [35]. Briefly, 1.0 μl of exponentially grown culture of *X. oryzae* (SR-PPXAP1) in PSB at 10^7 cfu/ml and phage suspension were mixed at a MOI of 0.1 and incubated at 28℃ for 10 min. Immediately after incubation, the mixture was diluted in such a way to minimize the chances of unabsorbed or released phages to infect new bacterial cells. The mixture was diluted to a final volume of 20 ml with PSB and incubated at 28℃. 100 μl samples were subsequently taken at 10 min interval up to 90 min and phage titer was determined by double agar method [33].

Determination of rate of adsorption of phage φXOF4: The rate at which the bacteriophages adsorps the host bacteria was determined as follows, a known volume containing phages (0.5 ml of 10^5 pfu/ml) was added to an equal volume of overnight grown culture of SR-PPXAP1 (10^6 cfu/ml) in a centrifuge tube and incubated at 28℃ for 10 min. The mixture was centrifuged at 1,900 ×*g* for 10 min. The resulting supernatant was assayed for phage on a sensitive indicator plate and the percentage adsorption was calculated based on the assay in the control (no bacteria).

Structural analysis of phage by TEM: A high titre phage lysate (10^9 to 10^{10} pfu/ml) was prepared in 10 ml of liquid culture and centrifuged at 75,000 rpm for 1 h at 4℃ [36]. The resulting pellet was dissolved in SM buffer. One drop of the phage suspension was placed onto a 300-mesh carbon coated grid and allowed to absorb for 1 min. The excess suspension was removed with filter paper kept at the edge of the grid. The grid was stained with 1% (w/v) potassium tungstate (pH 7.5) and the excess stain was removed in the same manner as stated previously. The specimen was viewed with transmission electron microscopy (TECHNAI-10, Philips, Netherland) at an acceleration voltage of 80 kV.

Thermal stability analysis of phage φXOF4: The thermal stability of phage φXOF4 was studied by exposing the mixture containing 900 μl of peptone sucrose broth (pH 6.8) and 100 μl of phage suspension at 2.8×10^9 pfu/ml to different temperatures 4, 28, 37, 50, 55, 60, 65, and 70℃ for 1 h. The mixture was cooled on ice and the intact phages were enumerated by double agar plate method described earlier.

Effect of pH on the stability of phage φXOF4: A mixture containing 900 μl of peptone sucrose broth (pH 6.8) and 100 μl of phage suspension at 2.8×10^9 pfu/ml was

exposed to different pH's 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 for 1 h at 28℃. The intact phages were immediately enumerated by double agar plate method as described previously.

Disinfection of *X. oryzae* with phages

Inoculation of *X. oryzae* onto the seeds: Inoculation of the seeds with streptomycin resistant *X. oryzae* bacterium (SR-PPXAP1) was carried out by following the methodology described by Kocharunchitt *et al.* [37] with little modification. The susceptible rice cultivar, TN1, seeds were used for this experiment. Five grams of rice seeds were immersed in 20 ml broth containing SR-PPXAP1 at 10^7 cfu/ml and incubated overnight at room temperature with gentle agitation. The supernatant containing unadsorbed bacteria was decanted and the infected seeds were washed with sterile distilled water twice and dried at room temperature (28℃) for 18–22 h. The seeds treated with 10 ml of sterile distilled water and processed in the same manner as described above served as the control.

Treatment of bacteriophage on to the infected seeds: Curative and protective forms of phage treatment were tried with the rice seeds. In the curative method, 5 g seeds inoculated with SR-PPXAP1 were taken in 100 ml conical flask and treated with 20 ml of phage φXOF4 at a particle density of 10^8 pfu/ml. The mixture was incubated at room temperature for 18–22 h with gentle agitation. The supernatant was decanted and seeds were kept in sterile distilled water to maintain constant moisture. The seeds soaked in water served as negative control without the bacterium (SR-PPXAP1). In the protective method, 5 g of TN1 seeds were immersed in 20 ml of phage φXOF4 suspension and incubated overnight with gentle agitation. The supernatant was decanted and 20 ml of suspension containing streptomycin resistant *X. oryzae* pv. *oryzae* at 10^7 cfu/ml was added and incubated at room temperature with gentle agitation for 1 h. Subsequently the supernatant was discarded and the seeds were soaked in sterile distilled water. The seeds soaked in phage suspension served as the control.

Effect of phage treatment on germinability and seedling vigor: The seeds of susceptible cultivar, TN1, treated with the test bacteria and phages alone or in combinations were used for the study. Fifty seeds drawn from each treatment, the cell suspension was drained off and the seeds were dried at room temperature. The dried seeds were surface sterilized and germinability of the seeds was checked using paper towel method [37]. The treated seeds were placed in paper towels (45 cm × 28 cm) soaked with sterile water. An additional presoaked paper towel was then placed on the first one to hold the seeds in position. The towels were then rolled and wrapped with polythene paper to prevent drying and incubated at 30° ± 2℃ for seven days. The paper towels were unrolled and the number of seeds germinated was recorded.

Enumeration of *X. oryzae* population after phage treatment: The germinated seedlings (3 seedlings/set) were taken from each set and ground with 10 ml of 1x PBS in a sterile mortar and pestle and 1.0 ml of homogenate was centrifuged at 10,000 rpm for 10 min at 4℃. The pellet was suspended in 1ml 1x PBS, serially diluted and plated on PSA amended with streptomycin (0.6 μg/ml). The Plates were incubated at 28℃ for 48–72 h. The number of colonies appeared was counted and the cfu/ml for each treatment were determined.

Phage titers during the challenge treatment were also determined. The supernatant from each treatment was filter sterilized with 0.22 μm filters, and serially diluted with SM buffer. Phage in the filtrate obtained from each sample was enumerated using SR-PPXAP1 as the host and spot test was done in order to determine the PFU/ml for each treatment.

Cultivation of rice plants: Seeds with various treatment processes were induced to germinate by incubating the seeds at 30℃ for 24 h. Earthenware pots (cm) were filled with vermiculite and granulated soil in the ratio of 1 : 2 and irrigated with sterile water. The germinated seeds were sown and covered with a thin layer of soil. The pots were initially covered with polyethene bags to facilitate emergence of seedlings and after that the cover was removed and the seedlings were grown for 7 days in under natural light conditions. Disease prevalence was scored after 7 days by counting the seedlings with disease symptoms and the ratio was calculated to the total number of seedlings examined.

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Results and Discussion

Isolation of *X. oryzae* pv. *oryzae*

One of the important criteria for designing the disease control strategies would be to identify the hotspots of infectious disease. Bacterial species in the hotspot region are considered to be more virulent and they exist in all seasons [38]. The details regarding the diversity of pathogen and structure of pathogen population are important when developing a strategy for plant disease management. Maruteru has been found to be one such hotspot for BLB in costal Andhra Pradesh where high disease incidence and disease severity have been observed in all the seasons [27]. The leaves showing typical symptoms of bacterial leaf blight were collected from the research field at Acharya N.G. Ranga Agricultural University (ANGRAU), Maruteru, Andhra Pradesh and farmer's field adjoining the University, during the month of December 2015. Isolation of *X. oryzae* from infected leaf sample was relatively easier than infected seeds as they known to harbor large population of bacteria and fungi on the surface [40] and hence in the present study the leaf samples were used for the isolation of *X. oryzae*.

A total number of 45 bacterial leaf blight infected rice leaf samples were collected from 15 different sites (Fig. 1) and as many as 19 different bacterial colonies were isolated. The isolated bacteria included the pathogenic *X. oryzae* as well as the fast-growing saprophytes associated with the rice leaves. Studies on bacterial populations associated with the rice plants have been traditionally focused on the bacteria of obvious interest, such as pathogen and biological control agents, however a large fractions of saprophytic and symbiotic bacteria are generally been neglected. Saprophytic bacteria have been included in current study as they also represent the community of organisms infecting the plant tissues. There were 12 Gram-negative and 7 Gram positive bacteria isolated in this study (Fig.2).

Pathogenicity testing of *X. oryzae* isolates

The pathogenicity of the isolated bacteria was evaluated by artificially inoculating them individually on to the leaves of susceptible rice cultivar TN1 under glass house conditions [13]. The development of typical water soaked lesions with wavy leaf margin which is characteristic of

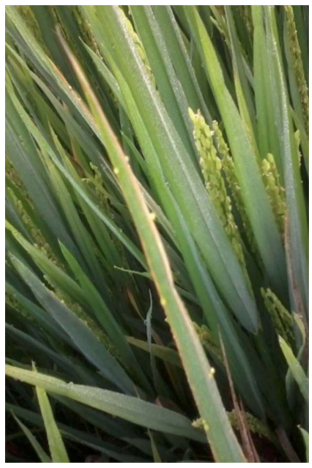

Fig. 1. Rice plant with characteristic Bacterial Leaf Blight disease symptoms.

X. oryzae infection was observed with 5 isolates such as PPXAP1, PPXAP2, PPXAP3, PPXAP4, and PPXAP5. These lesions turned whitish straw colour at later stages. A series of analysis showed that 5 of the 19 tested strains showed characteristic blight symptom.

Molecular identification of *X. oryzae* pv. *oryzae*

The PCR amplified product obtained from the DNA of all the 19 isolates with *X. oryzae* specific primers was analyzed on agarose gel (1%) electrophoresis. The appearance of 470 bp PCR amplified product on the gel revealed that they are *X. oryzae* pv. *oryzae* and 5 of the 19 isolated bacteria were identified as *X. oryzae* (Fig. 3).

A representative of the identified *X. oryzae* bacteria was grown on PSA (Peptone Sucrose Agar) medium which exhibited characteristic colony morphology as presented in Fig. 4 and the details of the identified bacteria are presented in Table 1.

Isolation of streptomycin resistant strain

The isolation and characterization of *X. oryzae* from rice plant and seeds by conventional technique are often hindered by the masking effect of fast growing saprophytic yellow pigmented bacteria and non availability of selective medium has further complicated the the isolation of *X. oryzae*. However, a semi selective medium has been developed containing trimethoprim which selectively inhibit the growth of saprophytic yellow pigmented bacteria. The semi selective medium did not inhibit the growth of all the saprophytic bacteria how-

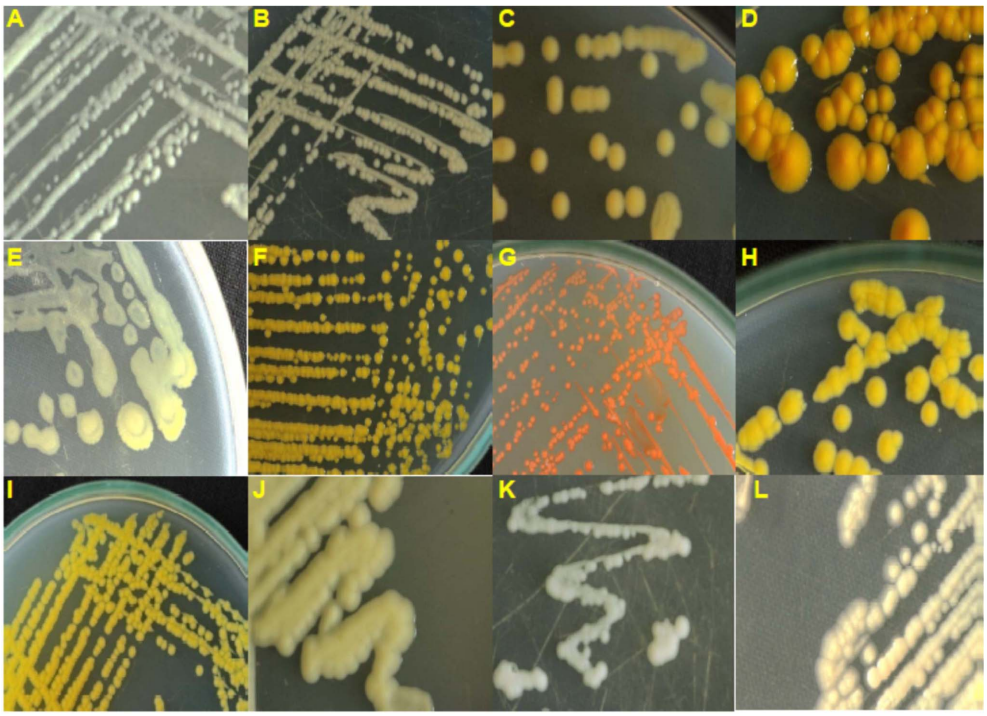

Fig. 2. Growth and morphological characteristics of bacteria isolated from infected rice plants.

ever it reduced the number of colony forming units. Hsieh *et al.* [40] developed an improved method for the detection of a streptomycin resistant isolates of *X. oryzae*, employing a selective medium incorporated with streptomycin. In the present study, streptomycin resistant bacterium was used to determine the exact number of bacteria after seed treatment with phages. The streptomycin resistant strain PPXAP1, grown well on PSA containing 100 µg/ml streptomycin (Sigma Aldrich) and the lesion length produced by this mutant was not significantly reduced (Fig. 6).

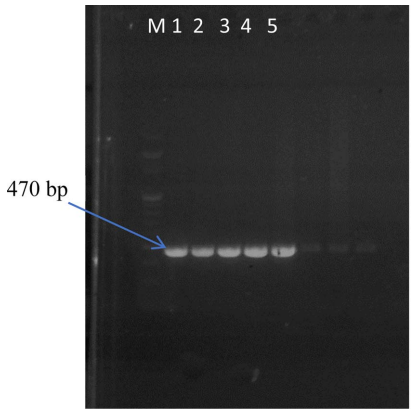

Fig. 3. PCR based identification of *X. oryzae* by amplification of 16S rRNA gene analyzed on agarose gel Lane M: Molecular size marker (100 bp DNA ladder). Lane 1-5, X.oryzae isolates (PPXAP1, PPXAP2, PPXAP3, PPXAP4, PPXAP5 respectively). The arrow indicates 470 bp amplication.

Isolation of bacteriophages

Phages are obligate parasites and are frequently encountered in close proximity with the bacteria. In the present investigation, soil and water samples collected

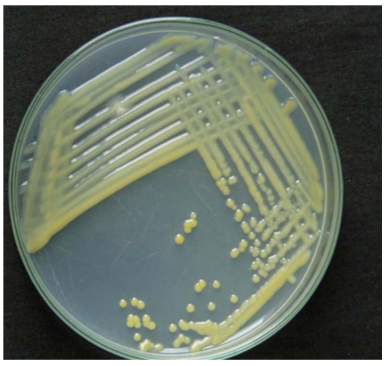

Fig. 4. Colony morphology of *X. oryzae* on Peptone sucrose Agar plate.

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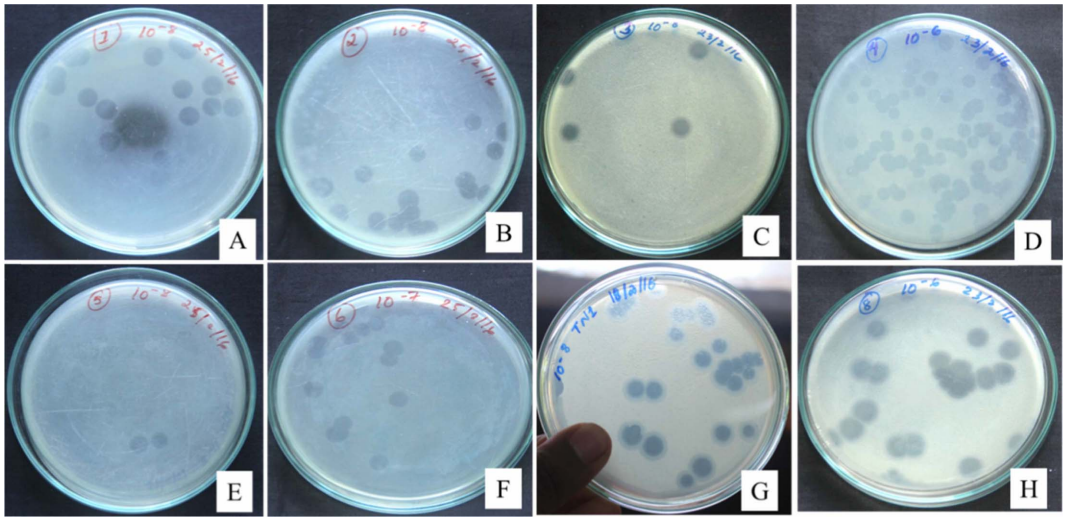

Fig. 5. Morphological analysis of plaques formed by isolated phages on susceptible *X. oryzae* (PPXAP1). (A) φXOF1, (B) φXOF2, (C) φXOF3, (D) φXOF4, (E) φXOT1, (F) φXOT2, (G) φXOM1, (H) φXOM2.

from the infected rice fields were used primarily as the source for bacteriophage isolation as per the method followed earlier by [41]. Five soil samples and 2 water samples from 15 sites were tested for the presence of phages specific for *X. oryzae*. In the present study, the direct isolation of phages from bacteria proved futile and therefore an enrichment method had to be adopted. The phage isolation medium was incorporated with PPXAP1 and PPXAP2 as host bacteria and using this enrichment method phages were detected in 3 of the 7 samples and there were 8 different bacteriophages obtained after two successive rounds of purification. Four phages were isolated from rhizosphere clay soil and remaining 4 phages were from irrigated water samples collected from rice fields infested with bacterial blight symptoms.

Plaque morphological analysis

The morphology of plaques formed by an individual bacteriophage on a susceptible bacterium is one of the important parameters for the characterization of phage [41]. The phages isolated in the present study were tested for bacteriolytic activity and plague formation on the lawn of susceptible PPXAP1. All the phages were able to lyse the PPXAP1 bacterium (Fig. 5). The sizes of the plaque formed by isolated phages were found to be between 2 to 5 mm. The size of plaque formed by a phage designated φXOM2 was found to be relatively larger (5 mm) when compared to the plaques of other phages (Table 2). However the diameter of the plaques increased further and formed a turbid zone around the lysed area when incubation time is increased to 36 to 48 h. The presence of a halo region could be due to the secretion of soluble enzyme by phages which could have degraded extracellular polymeric materials such as exopolysaccharides from the host strain [42]. All the isolated phages were treated with chloroform and were found to be resistant (Table 2) which indicated that viral coat not made up of lipid layer. The phages were adsorbed onto streptomycin resistant strain of bacterium (PPXAP1) grown on peptone sucrose agar medium which was incorporated with streptomycin (100 μg/ml) (Fig. 6). All the isolated phages were found to be resistant to streptomycin revealing that they are not sensitive to antibiotics.

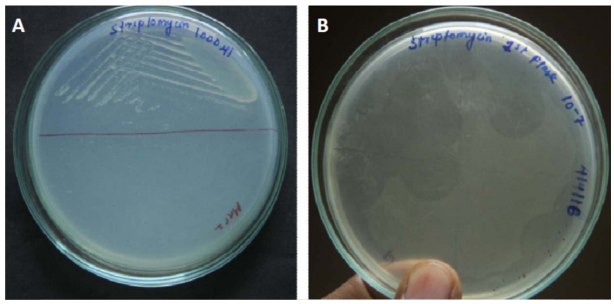

Fig. 6. Antibiotic resistance analysis of *X. oryzae* phage on streptomycin resistant *X. oryzae*. (A) Streptomycin resistant *X. oryzae*, (B) Lysis of streptomycin resistant *X. oryzae* by phage.

Determination of the emergence of Bacteriophage Insensitive Mutants (BIM)

It has been observed earlier that the development of resistance by bacteria against phages has been the major concern in the control of bacterial diseases. Therefore, an attempt has been made in the present investigation to ascertain whether bacteria develop resistance when treated with the isolated phages. A clear plaque is often indicative of the lytic nature of a phage while turbid plaque indicates temperate phages. Hence, experiment was conducted to identify the phages that induce no development of resistance in bacteria. Spot test was carried out with the phage lysates of all the 8 different phages on the lawn of PPXAP1 bacteria and incubated at 28℃ for 3–5 days. The bacterial colonies that emerge from the lysed area were observed. Emergence of bacterial lysogen was observed with all the isolated bacterial strains against all the isolated phages except φXOF4 which lysed all the bacteria completely resulting in clear plaque formation (Fig. 7). Notably, the bacteria in the lysed region did not grow when incubated for about one month. Hence, this phage was identified as the most virulent against all the isolated bacteria and chosen for bacterial infestation treatment studies in rice seeds.

Determination of host range for φXOF4 phage

One of the prerequisites of a phage that could be used for biological control of bacterial diseases is that it should not exert any lytic activity against the beneficial bacteria present in the phyllosphere and rhizosphere environment. Therefore, the lytic potential of φXOF4 phage was evaluated against the saprophytic bacteria using streak plate method. The bacteria were first streaked and 5 µl of phage lysate was added on the top of it and the plates were observed for the lysis of bacterial cells. The phage φXOF4 did not lyse any of the saprophytic bacteria obtained in the study and was found to be highly specific for the bacterium *X. oryzae*.

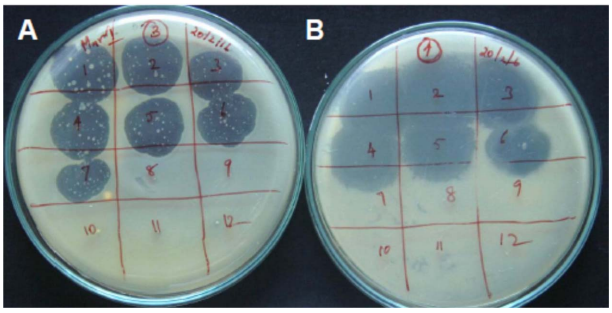

Fig. 7. Analysis of emergence of bacterial insensitive mutants against φXOF3 (A) and φXOF4 (B); 1-12 indicate phage dilution from 10 to 1.

Characterization of Bacteriophage φXOF4

Adsorption and one step growth curve studies: About 58% phage adherence occurred within 20 min of coincubation which increased to 70% when the incubation time was increased to 60 min at 28℃ (Fig. 8A). The one step growth curve analysis was carried out with phage φXOF4 to determine the replicative cycle of the virus. A triphasic curve including the latent, rise and plateau phase was obtained (Fig. 8B). It has been observed that

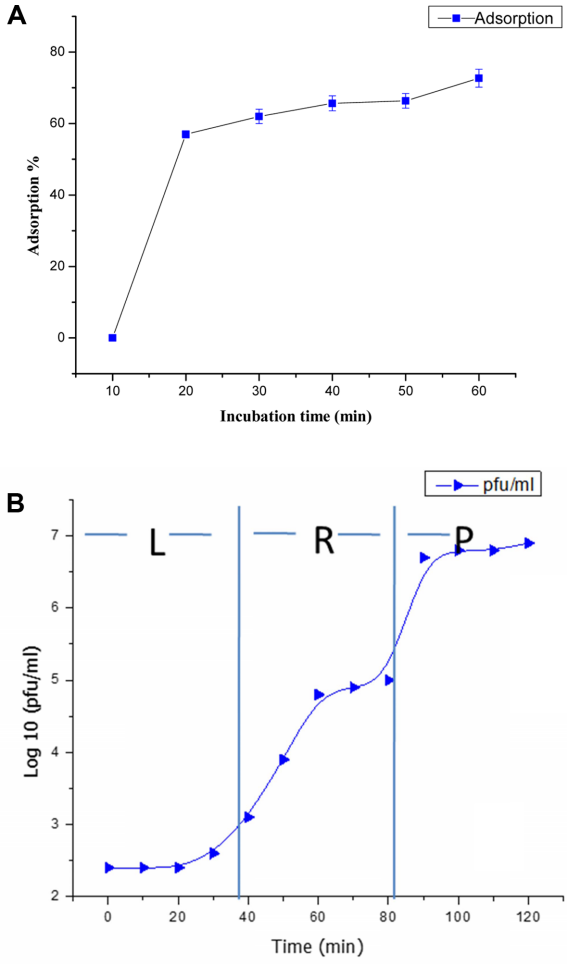

Fig. 8. Determination of rate of phage (φXOF4) adsorption on to PPXAP1 (A) and One step growth curve analysis for phage φXOF4 L: latent period; R: Rise period; P: Plateau period.

phage φXOF4 showed a shorter latent period, 20–30 min, and the rise period was about 60 min and the burst size was 1.8×10^7 pfu/ml. Phages with short latent period and high burst size are the most appropriate candidate for phage therapy [42].

Morphological characterization of phage φXOF4: In the present study the Transmission electron microscopic analysis revealed that phage φXOF4 containing icosahedral head with a tail. The diameter of the phage head was found to be 50 nm and the length of the tail was 40 nm (Fig. 9). According to the guidelines of International committee of Taxonomy of virus, the phages are classified based on the morphology into their viral orders and families. Based on the above characteristics, the phage was found to belong the order Caudovirales.

Effect of temperature and pH on the stability of φXOF4 phage particles: Several environmental factors such as temperature and pH contribute to the inactivation of phage particles by damaging their structural element [43]. Therefore, the stability of chosen phage φXOF4 at varying temperature and pH was investigated by determining the phage titer after treatment. The φXOF4 phage was found to be stable without considerable reduction in phage titer up to 50℃ for 1h after incubation (Fig. 10A). However, there was a sharp reduction in the phage titer when incubated at 55℃ and no phage titer was observed at 60℃ and beyond, suggesting the loss of phage viability at elevated temperatures. It has been suggested that the disulfide cross linkings in the phage capsid proteins are denatured at higher temperature leading to the loss of phage integrity [46].

The effect of acidic and alkaline environment on the stability of phage was studies in modified PS broth adjusted to pH 3–12 at 28℃. The phage was found to be stable at pH range 6 to 8 (Fig. 10B). The phage titer was drastically reduced when incubated at pH below 6 or above 8 and there was no sign.

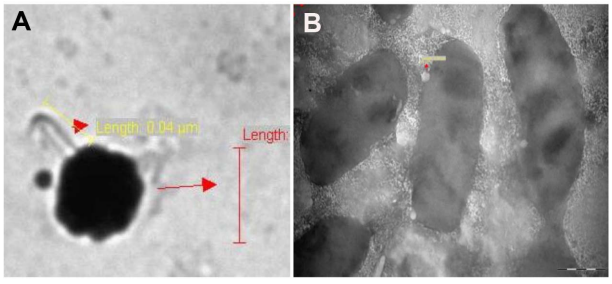

Fig. 9. Ultra structure (A) and attachment (B) of φXOF4 phage on to *X. oryzae* (scale 100 nm).

Effect of inoculum size of *X. oryzae* on the disease development

It has been established that the *X. oryzae* cell density influences development of blight disease in rice plants [45] and the effect of different inoculums sizes (1×10^1 through 1×10^{10} CFU/ml) on the development of disease on the seeds of susceptible rice cultivar, TN1, was evaluated [46]. The seedlings were observed for development of disease symptoms after 7 days of incubation. The disease incidence progressively increased as the concentra-

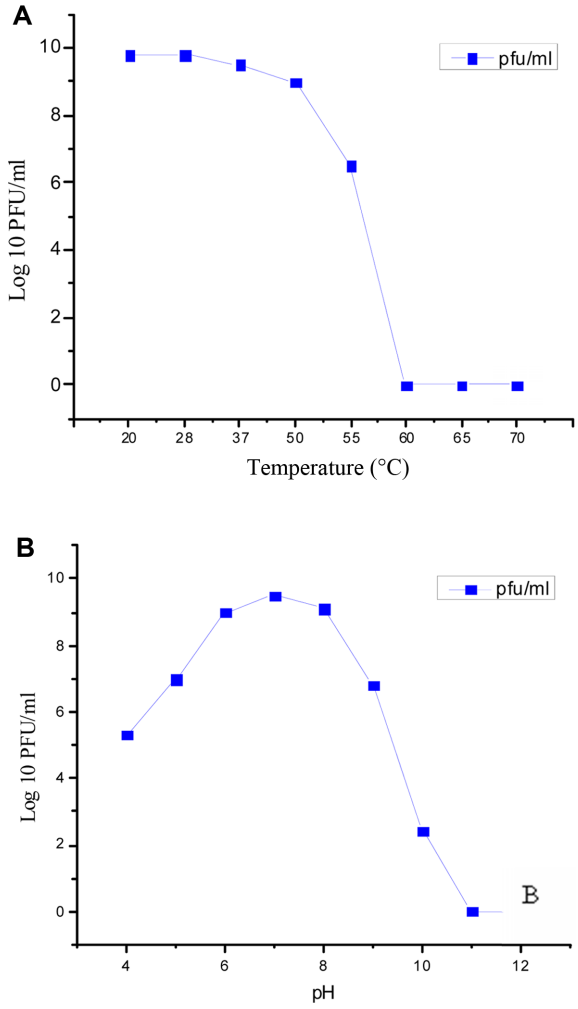

Fig. 10. Effect of temperature (A) and pH (B) on the stability of φXOF4 phage particles.

tion of the inoculum increased. About 50% of the seeds were infected when treated with an inoculum at 1×10^6 cfu/ml and at 1×10^7 cfu/ml 80% infection were observed (Fig. 11). Hence, to study the effect of seed treatment with phage lysate, a cell concentration of 1×10^7 cfu/ml of SR-PPXAP1 was used.

Effect of phage treatment on the germinability of rice seed

In order to verify if the treatment of seeds with phage lysate causes any adverse effect, the phage treated seeds were germinated in paper towels. The seeds treated with bacteria and phage alone and in combination did not have any effect on the germinability (Table 3). Interestingly, the germinability of seeds treated with phages alone was almost similar to the control seeds (Table 3).

Biological control of seed borne bacterial blight disease in rice

Almost all the food crops are propagated by seeds, the way by which embryonic life can be almost suspended and then revived into new plant even years after the parental generation. Therefore, production of pathogen free seeds assumes utmost importance in terms of nutritional and economic points of view. Disease free seeds reduce the chances of yield losses due to diseases caused by pathogenic organisms. The *in vitro* experiments were performed to determine the ability of phage φXOF4 to lyse *X. oryzae* adsorbed on to the rice seeds. When phage was added to the exponentially growing *X. oryzae* culture at a multiplicity of infection (MOI) of 10, the phage suppressed the growth of the pathogen resulting in the lysis of cells within an hour after application. Kocharunchitt *et al*. [46] observed that when single phage was added to exponentially growing *Salmonella* cultures at a MOI of approximately 70 resulted in lysis of bacteria immediately after application.

Table 3. Effect of phage treatment on germinability of rice seeds.

Treatment	Percentage seed germination occurred
Control	97
Bacteria alone	88
Bacteria + Phage	89
Phage alone	96
Phage + Bacteria	90

Table 4. Effect of phage treatment on the reduction of disease incidence.

Concentration of phage (φXOF4) used (pfu/ml)	Diseased incidence observed (%)
10^5	15
10^6	6.3
10^7	3.6
10^8	0
None	73

The effect of phage treatment on the reduction of disease incidence was carried out by treating the *X. oryzae* (SR PPXAP1) infected seeds with φXOF4 phage at different titres (10^5 to 10^8 pfu/ml). Complete inhibition of disease symptom appearance was observed in the seeds treated with 1×10^8 pfu/ml (Table 4) indicating that this particular phage titre is optimum for the elimination of the pathogen from the seeds. Reduced disease incidence was observed with seeds treated with 1×10^7, 1×10^6 and 1×10^5 pfu/ml as well where the disease incidence observed was 3.6, 6.3 and 15% respectively which clearly indicates that the phages are effective in controlling the blight disease with lesser phage titre. Earlier, the rice seeds treated with a phage designated BGPP-AR has significantly reduced the seedling rot disease incidence when used at 1×10^5 pfu/ml [15].

Following the *in vitro* trials, higher titer of phage φXOF4 at 1×10^8 PFU/ml was applied to infected seeds to evaluate its potential to control the pathogen. The phage did not eliminate *X. oryzae,* completely, however, the bacterial population was reduced to a significant level (Table 5). In each treatment the phage population increased when compared to the control (Phage alone) indicating the presence of host bacterium. The increased number of *X. oryzae* in experimentally contaminated rice seeds as attributed to the condusive environmental conditions during germination [46].

The recoverable population of phages in mono inocu-

Table 5. Initial and final count of bacteria and phage during and after treatment.

Days after inoculation	Bacterial (SR PPXAP1) population (cfu/ml)	Phage (φXOF4) population (pfu/ml)
0 day	1.65×10^6	1×10^8
7 days	1×10^9	3×10^5

http://dx.doi.org/10.4014/mbl.1807.07009

Table 6. Enumeration of *X. oryzae* (SR-PPXAP1) and phage (φXOF4) in 7 day old the seedlings.

Treatment	*X. oryzae* cfu/ml	φXOF4 pfu/ml
Control (water)	0	0
Bacteria alone	1×10^9	-
Phage alone	-	3×10^5
Bacteria + Phage	1.86×10^6	2.3×10^9
Phage + Bacteria	5.74×10^6	5.6×10^8

lated rice seeds decreased from 1×10^8 pfu/ml to 3×10^5 pfu/ml indicating the inability of phage to multiply in the absence of host bacterium (Table 6). The phage was found to be stable on both treated and control seeds as well as in their treatment solution during phage application. The phage population, however, increased from 1×10^8 to 2.3×10^9 after 7 days. Thus, it has been concluded that less number of phages in the host after mono-inoculation was probably due to absence of bacterial host receptors, whereas in the case of seeds pre inoculated with SR PPXAP1 higher phage population was observed. The recoverable population of *X. oryzae* (SR PPXAP1) from the seedlings increased from 1.65×10^6 to 1×10^9 cfu/ml. Unusual *X. oryzae* colony morphology in some samples recovered from the seeds treated with phages were also noted (Figure not included), these colonies were found to be relatively larger in size and was quite different when compared to the morphology of *X. oryzae* colonies recovered from the control samples.

Adachi and Tsukamoto [15] have previously observed that the rice seeds treated with phages have resulted in the inhibition of the bacterial pathogens responsible for seedling rot and blight diseases and this inhibition potential of the phage was found to be equivalent to that of chemical pesticides. Kauffman and Reddy, [40] have illustrated that the rise in bacteriophages play a role in reducing the disease incidence by declining the bacterial population in germinating seeds. Application of phages to disease infested seeds works as augmentation process there by reducing the bacterial number.

The observation made in the current study throws light on the application of bacteriophages as a potential biocontrol agent. The seeds treated with bacteriophages remained free from infection. This study has also revealed that phages are useful weapon for disinfecting the seeds, eliminating need for the application of hazardous chemicals and, above all, its stability is not questioned as it exists in close proximity with the pathogen in a controlled environment. The success of phage therapy relies on the selection of phagovars with wide host range. The host specificity of a phage is high and several different phagovars are known to exist even within a bacterial species, Therefore, further research is needed to identify other hot spots of BLB disease throughout the country and to isolate phages from those sites in order to obtain phages that exhibit broader host range.

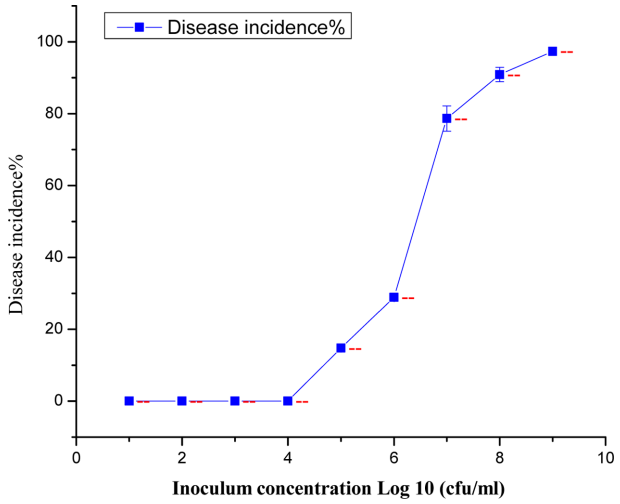

Fig. 11. Effect of inoculum concentration on the disease development by *X. oryzae* (SR-PPXAP1) in rice seeds.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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