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**Original Research Article**

# Management of Bacterial Leaf Blight Disease of Paddy (*Oryza sativa*) Caused by *Xanthomonas oryzae* pv. *oryzae* Using Bacteriophages

**S. Chandraprakash[1*], K. Manonmani[2], N. Revathy[3] and E. Kokila Devi[4]**

[1](Pl. Path.), Ramakrishna Mission Vivekananda Education and Research Institute (RKMVERI), Coimbatore – 641 020, Tamil Nadu, India
[2](Pl. Path.), Krishi Vigyan Kendra, Agriculture College and Research Institute (TNAU), Madurai – 625 104, Tamil Nadu, India
[3](Pl. Path.), Department of Plant Pathology, Agriculture College and Research Institute (TNAU), Madurai – 625 104, Tamil Nadu, India
[4](Pl. Biotechnology), Department of Biotechnology, Agriculture College and Research Institute (TNAU), Madurai – 625 104, Tamil Nadu, India
**Corresponding author*

## A B S T R A C T

*Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is the pathogen causing bacterial leaf blight (BLB) disease in paddy crop and its occurrence was observed in severe form in paddy fields of Madurai during *Kharif* and *Rabi* seasons of 2016. Survey on the severity of BLB disease was conducted in 11 different places of Madurai district. From the BLB infected samples collected in these areas, 11 isolates of the pathogen, *Xoo* were isolated and characterised by standard morphological and molecular methods. All the 11 isolates of *Xoo* were virulent, by expressing differences in disease intensity on susceptible paddy cultivar, ADT 39 inoculated with clip inoculation method. The isolate *Xoo*M1 was found to be virulent and used in further study. Bacteriophages were isolated from the paddy field flood water and BLB infected leaf samples. Totally five isolates of bacteriophage was recovered by three successive single plaque isolations by using *Xoo* as host. Among these five, *Xoo* Bp (MDU 1) was found to be highly effective in relative plaque forming efficiency *in vitro* and recovering viable count of *Xoo* M1 after 120 min of incubation. Then it was examined using a Transmission Electron Microscope (TEM) under 20 nm and 50 nm magnification and the size of the phage head was 61.8 x 55.7 nm and length of tail was 95.9 nm. Phage *Xoo* Bp MDU1 was formulated in different protectants *viz.*, pregelatinised corn flour (PCF – 0.5% and 0.25%), Skim milk (0.05% and 0.75%), Sucrose (0.5%), Gluten (1%), Gelatin (0.5%) and in combinations. Likewise 11 formulations were prepared in combinations of the protectants and tested for their effect on BLB disease management. Among these 11 formulations, phage titer was found to be highest in skim milk (0.5%) + sucrose (0.5%) formulation and the phage titre was 9.85 $x10^{10}$ PFU/ml. The skim milk (0.5%) + sucrose (0.5%) formulation recorded the lowest PDI of 33.58 with a percent reduction over control (PROC) of 40.73. Under artificially inoculated condition, seed treatment with skim milk formulation of *Xoo* Bp (MDU1) @ 200ml/kg and foliar spray @ 20% recorded PDI of 37.5 and 24.40 PROC.

**Keywords**

Paddy, Bacterial Leaf Blight, Bacteriophages

## Introduction

Rice is the most important and extensively grown food crop in the World. Rice production is mostly affected by pathogens causing fungal, bacterial and viral diseases. Bacterial leaf blight of rice (BLB) caused by *Xanthomonas oryzae* pv. *oryzae (Xoo)* (Swing *et al.,* 1990**)** is one of the major diseases of rice globally in the irrigated agro ecosystem.

It is one of the most devastating diseases of rice causing significant yield reduction under serious infestations in many rice growing countries. In India, this disease is prevalent in almost all paddy growing regions in the state. It was first reported from India by Srinivasan *et al.,* (1959). The disease incidence ranges from 70 to 80% leads to significant crop damages (Sere *et al.,* 2005; Basso *et al.,* 2011).

In Tamil Nadu, both the outbreak and severity of the disease are not uniform at all the rice belts as has been observed in the past. For management of the disease, no effective chemical could be recommended for practical use (Ou 1985; Reddy and Nayak, 1985) for the reasons that these chemicals are effective only when applied just at the initial stage of the disease; the chemicals possess their inherent ill effects like prompting the pathogen for developing resistant strains; residual toxicity problem and above all causing environmental pollution.

The development of biological control methods may be a good complement to manage the disease and many biological control agents have been described in the past several years (Elmer and Reglinski, 2006; Ajouz *et al.,* 2011). Recently, there has been an increased interest in the use of bacteriophages for control of bacterial plant diseases (Flaherty *et al.,* 2000). Phages were discovered in the early part of the twentieth century by Twort in 1915 and by d'Herelle in 1917 (Summers, 2005), who independently reported about filterable and transmissible agents of bacterial lysis.

They did not agree, on the origin of this "lytic principle." Twort proposed that a bacterial enzyme with ability to grow caused the lysis, d'Herelle speculated that a virus was responsible for the phenomenon. Hence, this study aimed on isolation of bacteriopahges from paddy ecosystem and assessed their efficacy in managing bacterial leaf blight disease of paddy.

## Materials and Methods

The present investigations on 'Management of bacterial leaf blight disease of rice (*Oryza sativa*) caused by *Xanthomonas oryzae* pv. *oryzae* using biocontrol agents' were carried out in laboratory and glasshouse at the Department of Plant Pathology, Agricultural College and Research Institute, Madurai, which is situated at 9°54' N latitude and 78°54' E longitude and at an elevation of 147m above MSL.

### Assessment of bacterial leaf blight (BLB) disease incidence in paddy growing areas of Madurai District

Roving survey was conducted to assess the incidence of rice bacterial leaf blight (BLB) disease in different villages representing various blocks of Madurai district during *Kharif* and *Rabi* seasons of 2016. The disease grade for the infected leaves was assigned as per the standard grade chart (IRRI, 1988; 1989).

The per cent disease indices (PDI) were calculated using the following formula (McKinney, 1923)

PDI = (Sum of all numerical ratings / Total number of leaves graded) × (100 / Maximum grade)

## Isolation of pathogen from diseased leaf samples

Bacterial leaf blight infected paddy leaves were collected from the above 11 locations of Madurai district and the diseased leaf bits of 2x7 mm size were surface sterilized for 10 seconds with 0.1% mercuric chloride and washed in two changes of sterile water. Then the pure culture of 11 isolates of *Xanthomonas oryzae* pv. *oryzae* (*Xoo)* were isolated, purified and preserved at 4°C by transferring inoculum into screw capped bottles containing sterile distilled water separately (Wilson *et al.,* 1993).

## Screening of virulent isolate of *Xoo*

Inoculum of each isolate of the BLB causing pathogen, *Xoo* was prepared by streaking a loopful of an isolate in the middle of NA plates and incubated at 25-27°C. The bacterial growth was washed off from the plate surface after 24 h with 5 ml of sterile distilled water.

The inoculum thus prepared was serially diluted and adjusted to a concentration of $10^7$- $10^8$ colony forming unit (CFU) per ml**.** Based on the susceptible nature to BLB, paddy cultivar ADT- 39 was used for pathogenicity test by using leaf clipping method by maintaining three replications in completely randomized design (Klement and Goodman, 1967).

## Phage isolation and purification

Virulent isolate of *Xoo*, was used as hosts for the isolation of phages from flood water samples of paddy field and BLB infected paddy leaves (Balogh, 2006).

## Screening of efficient phage against the virulent isolate of *Xoo*

## Plaque forming efficiency of phages *in vitro*

Five different phages *viz.*, *Xoo* Bp (MDU1) and *Xoo* Bp (MDU2), *Xoo* Bp (MDU3), *Xoo* Bp M1 and *Xoo* Bp S1 were used. Each phage stock was diluted to $10^5$ PFU/ml, 30 µl of the phage suspension and 100 µl of the cells from an overnight culture of *Xoo* ($10^8$ CFU/ml) were mixed with three ml of the molten soft Peptone sucrose agar (PSA) medium and poured onto the surface of a regular PSA plate separately. The plaques were differentiated according to the degrees of clarity and sizes after incubation at 28°C for 18 h. (Chae *et al.,* 2014).

## Effect of phages on virulent isolate of *Xoo in vitro* (Viable bacterial count)

A bacterial suspension containing 2.5 ×$10^7$ CFU/ml of *Xoo* in five ml of Peptone Sucrose (PS) broth was incubated with phage isolates *Xoo* Bp (MDU1) and *Xoo* Bp (MDU2), *Xoo* Bp (MDU3), *Xoo* Bp M1 and *Xoo* Bp S1 in shaking culture separately. To determine the number of viable bacteria, portions of the suspensions were serially diluted, plated onto NA and the agar plates were incubated at 37°C for 24 h. Viable bacterial count was taken after 120 min. (Watanabe *et al.,* 2007).

## Transmission Electron Microscopy

One drop of high-titer phage suspension was placed onto a 300- mesh nickel grid coated with formvar, and the phage, *Xoo* Bp (MDU1) on the grid were negatively stained with 2% uranyl acetate or 3% sodium phosphotungstate. Each sample was examined using a FEI, Tecnai sprit G2 (Netherland) transmission electron

microscope (TEM) with an accelerating voltage of 120 kV (Chae *et al.*, 2014).

## Development and application of protective formulation of virulent phage for managing BLB disease under artificially inoculated condition

The virulent isolate of *Xoo* M1 was inoculated onto the leaves of rice plants (ADT 39) before panicle initiation stage by clip inoculation method and the pots were maintained at 27°C and 70% RH. The virulent phage *Xoo* Bp (MDU1) was mixed with protective formulations *viz.*, pregelatinised corn flour (PCF – 0.5% and 0.25%), Skim milk (0.05% and 0.75%), Sucrose (0.5%), Gluten (1%), Gelatin (0.5%). Likewise 11 formulations were prepared in combinations of the protectants and tested for their effect on BLB disease management. The formulations were delivered onto the leaves of rice plants after two days of inoculation of the virulent isolate of *Xoo* at a concentration of $10^8$ PFU/ml. The formulations were applied with hand - held sprayers in the morning hours and one more spraying was done in seven days interval and three replications were maintained (Balogh, 2002).

## Phage recovery

Leaf samples located on exposed positions of the plants were collected from the treatments on 4 days after phage application and samples were placed in plastic freezer bags and after the addition of 50 ml sterilized tap water, the samples were shaken for 20 min. Two milliliters were collected and centrifuged at 10,000 g for 10 min to remove debris. Then 100µl chloroform was added to each microcentrifuge tube and the tubes were incubated on a rotary shaker for 30 min. Following incubation, the chloroform was pelleted by a pulse-spin in a microcentrifuge and 700 µl of the supernatant was transferred into a sterile microcentrifuge tube. The tube was centrifuged for 15 minutes at 14000 rpm in order to remove cellular debris. The supernatant was used for phage recovery and enumeration of the phage titer.

## Assessment of inhibition of virulent isolate of *Xoo* by phage *Xoo*Bp MDU1

The 24 h old bacterial culture was removed from agar plates and suspended in sterile tap water. A 100-µl aliquot of the phage suspension was placed in a sterile Petri dish followed by the addition of 100 µl of the suspension of the virulent isolate of *Xoo*. Finally, 16 ml of sterilized NYA medium heated to 48-50°C, was added. L rod was rotated in Petri dish to facilitate thorough mixing of bacterial cells and the phages. The plate was incubated at 28 °C for 2-3 days until the bacterial lawn appeared and the plaques were observed. (Balogh, 2002).

## Enumeration of phage titer

Following phage recovery, the plaques were counted at the suitable dilutions. The phage titer was expressed as number of plaque forming units (PFU) per gram leaf tissue by the following equation: y = plaque number x 1000 (since 100 µl of the original 100-ml volume was plated) / dilution ratio/weight of leaf sample (Balogh, 2002). BLB intensity was assessed following the Standard Evaluation System (SES) for rice (IRRI, 1988; 1989) in 20 randomly selected leaves on 75 DAT.

## Efficacy of phages on BLB incidence in paddy under artificially inoculated conditions

Rice seeds (ADT 39) were soaked in double the volume of sterile distilled water containing the required quantity of

formulations of various phages as detailed in Table 6. After 24 h, the suspension was drained off and the seeds were dried under shade for 30 min. Treated seeds and untreated control seeds were sown in plastic pots separately.

The seedlings were transplanted in plastic pots containing 3kg of soil at the rate of two seedlings per hill and four hills per pot (Nandakumar *et al.,* 2001) and were watered regularly to maintain one cm water level.

Virulent isolate of *Xoo* was inoculated by leaf clipping method of inoculation.

Foliar application of formulations of phages and chemical treatments were given on next day of pathogen inoculation and repeated seven days after first spray.

Foliar spray with Copper hydroxide (Kocide) 2.5% and foliar spray with COC 0.25% + Streptomycin 100ppm served as a chemical check.

Three replications were maintained for each treatment in completely randomized design under glasshouse conditions.

The effectiveness of the treatments on managing BLB disease intensity was recorded on 75 days after transplanting (DAT), with a 0-9 scale of the Standard Evaluation System for rice (IRRI) and PDI was calculated.

### Statistical analysis

The data were subjected to statistical analysis following the method of variance described by Gomez and Gomez (1984).

Least significant difference (LSD) at 5 % level was calculated to determine significant differences between treatments.

## Results and Discussion

### Occurrence of Bacterial leaf blight (BLB) disease of paddy and pathogen variability in Madurai district

A roving survey was conducted to assess the incidence of bacterial leaf blight (BLB) disease in different rice growing areas of Madurai district.

The disease index was ranged from 40.00 to 63.88 per cent and the maximum index of 63.88 per cent was recorded in Mangulam village. The minimum disease index of 40.00 per cent was recorded at Pudhupatti village (Table 1; Plate 1).

Variation in BLB disease intensity might be due to the weather factors and microclimatic conditions of paddy ecosystem, varietal diversity in the areas surveyed and variability among the isolates of the pathogen, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*).

Regarding varietal diversity, many high yielding and nutrient responding varieties were susceptible to pests and disease and such varieties attributed the outbreak of BLB disease recent years in Madurai district.

The pathogen was isolated from BLB infected leaves showing marginal yellowing. The bacterial exudates from fresh lesion are better isolation material as compared to infected tissue because of less contamination (Isaka, 1970).

Similarly, Di *et al.,* (1991) reported that recovery of *Xoo* colonies from infected leaves sample is easy rather than infected seeds, due to the presence of other strains of bacteria and fungi in high population in seeds.

**Table.1** Bacterial Leaf Blight (BLB) disease incidence in paddy fields of Madurai district

| S. No. | Locations | ID of the *Xoo* isolates | Per cent disease index (PDI)* |
|---|---|---|---|
| 1 | Mangulam | *Xoo*M1 | 63.88 [a] (53.07) |
| 2 | Kulamangalam | *Xoo*K1 | 55.55 [b] (48.19) |
| 3 | Narasingam | *Xoo*N1 | 48.33[e] (44.04) |
| 4 | Vadipatti | *Xoo*V1 | 51.11[cd] (45.64) |
| 5 | Melur | *Xoo*M2 | 49.44[de] (44.68) |
| 6 | Thirumangalam | *Xoo*T1 | 43.88[f] (41.48) |
| 7 | Alanganallur | *Xoo*A1 | 61.66[a] (51.75) |
| 8 | Boodhakudi | *Xoo*B1 | 41.11[g] (39.88) |
| 9 | Saruguvalaiyapatti | *Xoo*S1 | 52.77[c] (46.59) |
| 10 | Pudhupatti | *Xoo*P1 | 40.00[g] (39.23) |
| 11 | Chekkanurani | *Xoo*C1 | 42.22[fg] (40.53) |
| | | **CD (P = 0.05)** | **2.42** |

*Mean of three replications
Values in parentheses are arcsine- transformed values
Figures followed by same letters are not significantly different

**Table.2** Screening of virulent isolate of *Xoo* causing BLB disease in paddy under artificially inoculated condition (Clip inoculation)

| S. No. | Isolate ID. | PDI* | Virulency |
|---|---|---|---|
| 1 | *Xoo*M1 | 74.44[a] (59.64) | Highly virulent |
| 2 | *Xoo*K1 | 65.55[c] (54.07) | Virulent |
| 3 | *Xoo*N1 | 52.22[f] (46.26) | Virulent |
| 4 | *Xoo*V1 | 54.44[e] (47.57) | Virulent |
| 5 | *Xoo*M2 | 53.33[ef] (46.91) | Virulent |
| 6 | *Xoo*T1 | 49.99[g] (45.00) | Virulent |
| 7 | *Xoo*A1 | 71.10[b] (57.49) | Highly virulent |
| 8 | *Xoo*B1 | 43.33[i] (41.17) | Virulent |
| 9 | *Xoo*S1 | 56.66[d] (48.56) | Virulent |
| 10 | *Xoo*P1 | 42.22[i] (40.53) | Virulent |
| 11 | *Xoo*C1 | 45.55[h] (42.46) | Virulent |
| 12 | Control | 0.0[j] (0.48) | - |
| | **CD (P=0.05)** | **1.5** | - |

*Mean of three replications
Values in parentheses are arcsine- transformed values
Figures followed by same letters are not significantly different

**Table.3** Isolates of Bacteriophages collected from Madurai district

| S. No. | Locations | Sources | Isolate ID. |
|---|---|---|---|
| 1 | AC& RI, Madurai | Paddy field water | *Xoo* Bp (MDU1) |
| 2 | AC& RI, Madurai | Paddy leaves | *Xoo* Bp (MDU2) |
| 3 | AC& RI, Madurai | Paddy field water | *Xoo* Bp (MDU 3) |
| 4 | Melur | Paddy leaves | *Xoo* Bp M1 |
| 5 | Saruguvalaiyapatti | Paddy field water | *Xoo* Bp S1 |

**Table.4** Plaque forming efficiency of phages on virulent isolate of *Xoo* M1

| S. No. | Isolate ID. | Plaque diameter (mm) * | No. of plaques* | Plaque type |
|---|---|---|---|---|
| 1 | *Xoo* Bp (MDU1) | $2.0^{c}$ | $138.45^{a}$ (2.14) | Clear |
| 2 | *Xoo* Bp (MDU2) | $1.8^{d}$ | $87.38^{b}$ (1.95) | Clear |
| 3 | *Xoo* Bp (MDU 3) | $1.8^{d}$ | $35.76^{d}$ (1.99) | Clear |
| 4 | *Xoo* Bp M1 | $2.2^{b}$ | $45.34^{c}$ (1.55) | Turbid |
| 5 | *Xoo* Bp S1 | $2.4^{a}$ | $38.21^{d}$ (0.58) | Clear |
| 6 | Control | - | 0 | - |
| **CD (P= 0.05)** | | **0.10** | **0.013** | |

* Mean diameter of ten plaques
Values in parentheses are log $_{10}$ transformed values
Figures followed by same letters are not significantly different

**Table.5** Effect of bacteriophages on virulent isolate *Xoo* M1 *in vitro* by viable bacterial count

| S. No. | Isolate ID. | After 120 min. of incubation | |
|---|---|---|---|
| | | **No. of viable bacteria (x $10^5$ CFU)/ml** | **Per cent reduction over control** |
| 1 | *Xoo* Bp (MDU1) | $0.13^{f}$ | 94.8 |
| 2 | *Xoo* Bp (MDU2) | $0.56^{e}$ | 77.6 |
| 3 | *Xoo* Bp (MDU 3) | $0.86^{c}$ | 65.6 |
| 4 | *Xoo* Bp M1 | $0.74^{d}$ | 70.4 |
| 5 | *Xoo* Bp S1 | $0.95^{b}$ | 64.0 |
| 6 | Control | $2.5^{a}$ | - |
| | CD(P=0.05) | **0.03** | |

*Mean of three replications
Figures followed by same letters are not significantly different

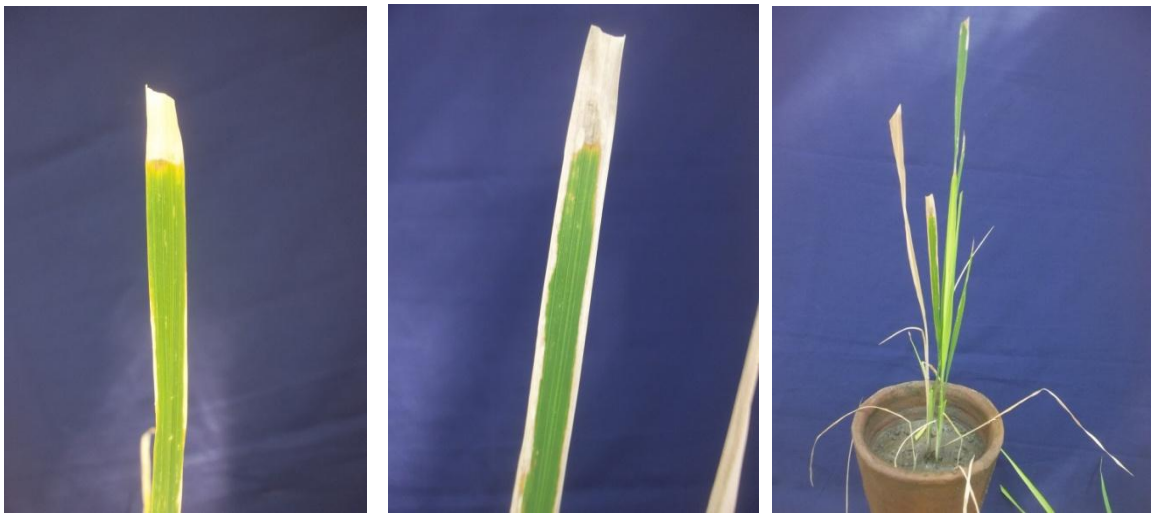
**Table.6** Efficacy of various protective formulations of phage *Xoo* Bp MDU1 against the virulent isolate of *Xoo* M1 under artificially inoculated condition

| S. No | Treatment Number | Treatments | Phage titer* (x$10^{10}$ PFU/ml) | PDI* | Percent reduction over control |
|---|---|---|---|---|---|
| 1 | T1 | Pre gelatinized corn flour (PCF) (0.5%) | 8.86$^{cd}$ | 39.30$^{fg}$ (38.83) | 30.64 |
| 2. | T2 | PCF (0.5%) + Sucrose (0.5%) | 9.35$^{b}$ | 36.66$^{h}$ (37.27) | 35.30 |
| 3. | T3 | PCF (0.25%) + Gluten (0.25%) + KOH (0.05%) | 6.76$^{g}$ | 45.54$^{d}$ (42.44) | 19.63 |
| 4. | T4 | Skim milk (0.5%) | 8.74$^{d}$ | 38.37$^{gh}$ (38.27) | 32.29 |
| 5. | T5 | Skim milk (0.5%) + Sucrose (0.5%) | 9.85$^{a}$ | 33.58$^{i}$ (35.41) | 40.73 |
| 6. | T6 | Skim milk (0.5%) + Gelatin (0.5%) | 7.56$^{f}$ | 42.24$^{e}$ (40.54) | 25.45 |
| 7. | T7 | Skim milk (0.75%) | 8.34$^{e}$ | 40.30$^{f}$ (39.41) | 28.87 |
| 8. | T8 | Skim milk (0.75%) + Sucrose (0.5%) | 9.02$^{c}$ | 37.32$^{h}$ (37.65) | 34.13 |
| 9. | T9 | Skim milk (0.75%) + Gelatin (0.5%) | 5.36$^{h}$ | 43.31$^{e}$ (41.16) | 23.56 |
| 10. | T10 | Gluten 1% | 4.84$^{i}$ | 45.67$^{d}$ (42.52) | 19.40 |
| 11. | T11 | Gelatin (0.5%) | 4.26$^{j}$ | 49.24$^{c}$ (44.53) | 13.15 |
| 12. | T12 | Non formulated | 2.43$^{k}$ | 52.32$^{b}$ (46.33) | 7.66 |
| 13. | T13 | Sterile water | 0.00$^{l}$ | 56.66$^{a}$ (48.83) | - |
| 14. | T14 | Foliar spray with COC 0.25% + Streptomycin 100ppm | - | 22.50$^{j}$ (28.31) | 60.29 |
| **CD(P=0.05)** | | | **0.27** | **1.84** | **-** |

*Mean of three replications
Values in parentheses are arcsine- transformed values
Figures followed by same letters are not significantly different

**Plate.1** Assessment of bacterial leaf blight (BLB) disease incidence in paddy field of Madurai district

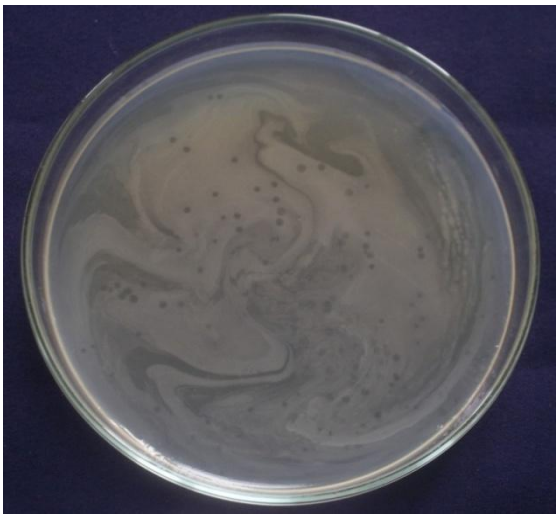
**Plate.2** Effect of different methods of inoculation of *Xoo* isolates on the ADT 39 paddy plants under artificially inoculated condition



Clip inoculation

**Plate.3** Morphological characters of phage isolates

*Xoo* Bp (MDU1)



*Xoo* Bp (MDU2)

**Plate.4** TEM images of *Xoo* Bp (MDU1)

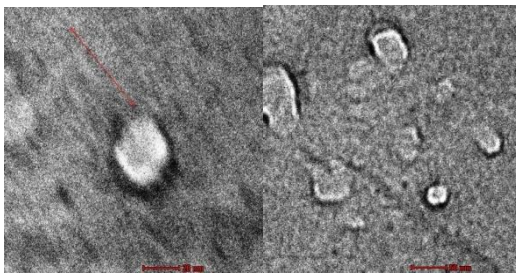


Image in 20nm Image in 50nm

**Plate.5** Relative plaque forming efficiency of phages on *Xoo* M1

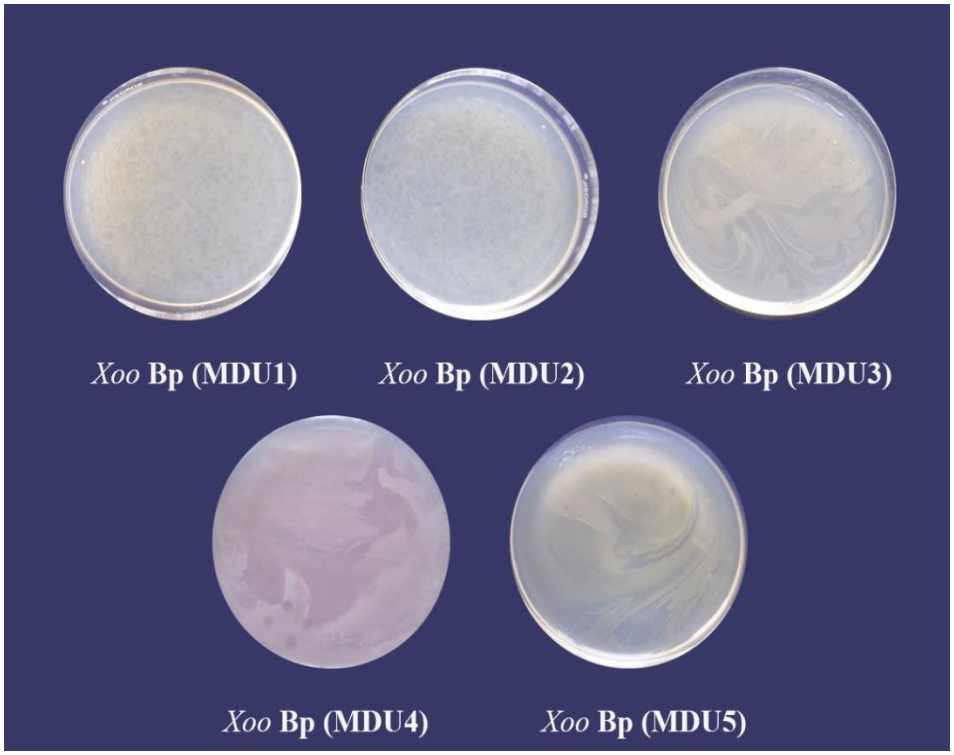



**Plate.6** Effect of bacteriophage isolates on *Xoo* M1 *in vitro*

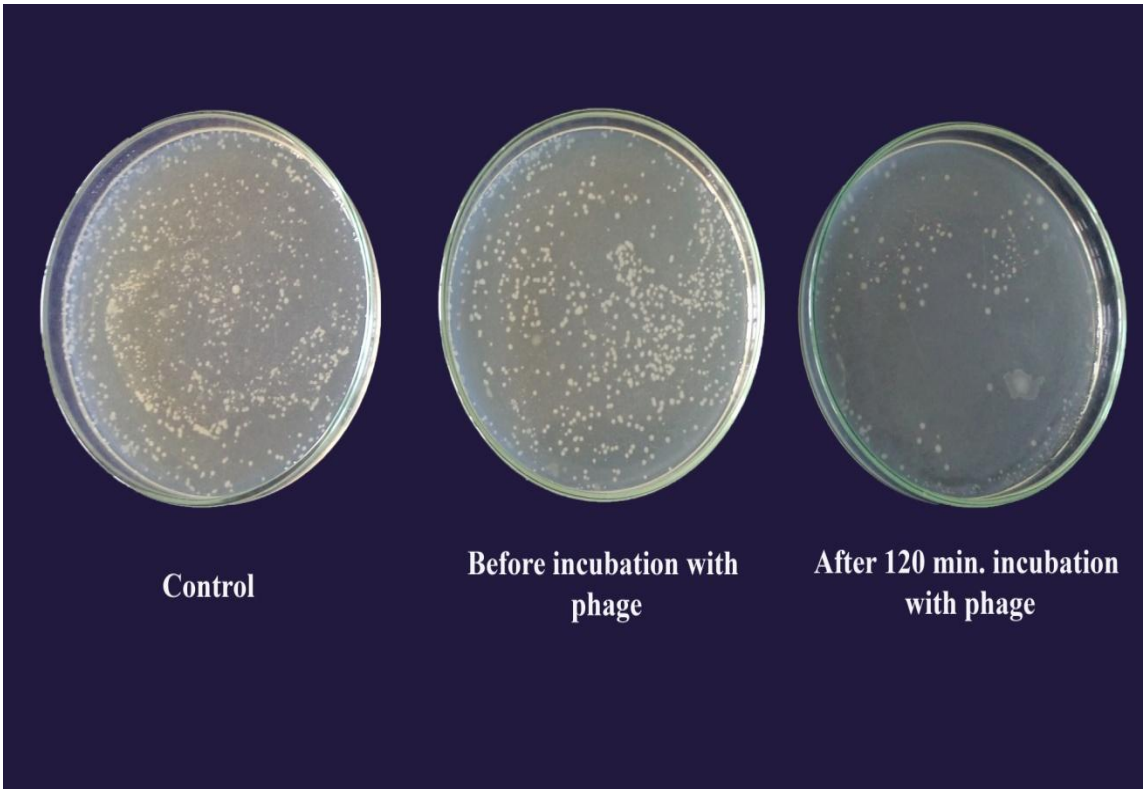
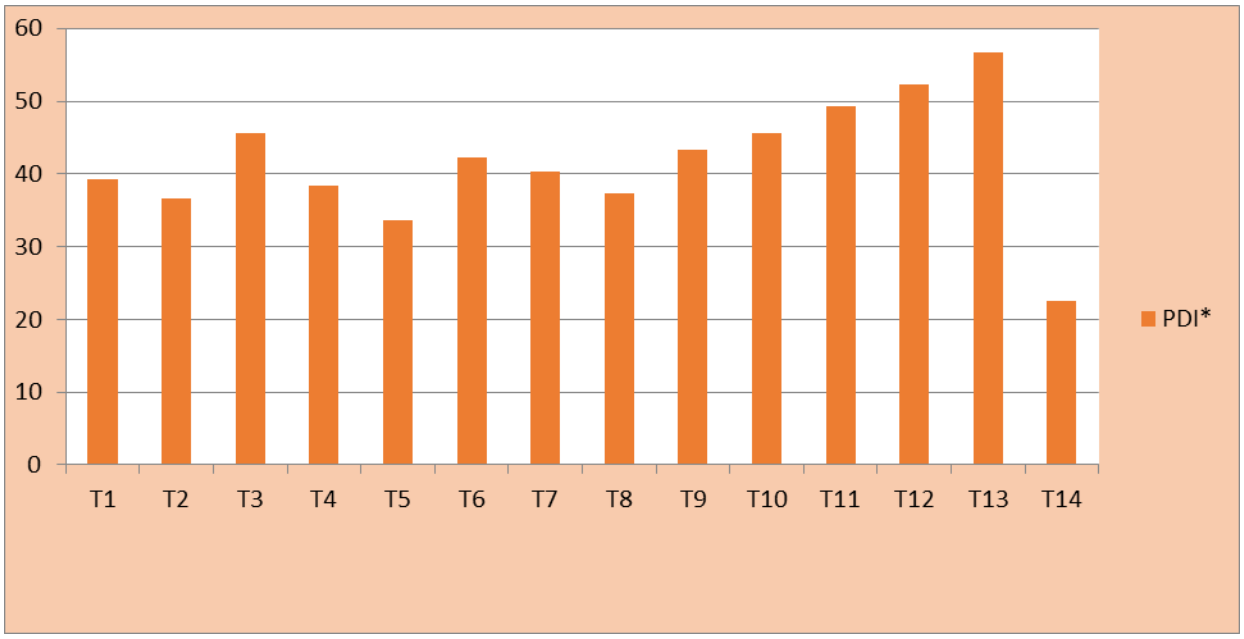


**Fig.1** Efficacy of various protective formulations of phage *Xoo* Bp MDU1 against the virulent isolate of *Xoo* M1 under artificially inoculated condition




Variability in the 11 isolates of the pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) purified from the BLB infected paddy leaves was observed. Likewise isolates of *Xoo* obtained from infected foliar samples of paddy collected from different agro ecological zones of Pakistan exhibited variation in culturing and pathogenecity (Jabeen *et al.*, 2012) and a survey conducted in nine different locations of Punjab yielded nine different isolates of *Xoo* differing in their ability to manifest pathogenic interaction with paddy (Arshad *et al.*, 2013). Parthasarathy *et al.*, (2014) reported the plant pathogens were isolated from the infected paddy plant collected from the field in Aduthurai, Thanjavur District and found to exhibit variation in culturing.

## Virulence of *Xoo* isolates

In the present study, all the 11 isolates of *Xoo* were tested for pathogenicity using ADT 39 as susceptible host of BLB by clip inoculation method and the results indicated that the isolate *Xoo* M1 was the most virulent by recording PDI of 74.44, followed by isolates *Xoo* M1 and *Xoo* A1 were highly virulent. The isolate *Xoo* P1 was the least virulent one with a lowest PDI of 42.22 (Table 2; Plate 2). Likewise, Arshad *et al.*, (2013) conducted *Xoo* virulent test on the rice Basmati super and Basmati 2000.

All these elevan isolates of *Xoo* were widely distributed in paddy growing areas of Madurai district and all were pathogenic. From these infected leaves, the isolates of *Xoo* were reisolated and maintained as a pure culture for further study.

Clipping method of artificial inoculation helps in establishing pathogenic interaction of *Xoo* with the host because the bacterial cells might have get deposited directly on the cut ends of xylem vessels, where the pathogen manifested itself in the host systemically and caused the lesion development within five days after artificial inoculation.

The variation in BLB incidence was due to the different types of inoculation methods. Akhtar *et al.,* (2008) conducted the three types of inoculation methods *viz.,* clipping, pin prick and brush methods and observed the best lesions produced by *Xoo* in clipping method. Similar work was reported by Rafi *et al.,* (2013) on the basis of colony morphology, 125 isolates were tested for pathogenicity using JP-5 as susceptible host of BLB. Bharathkumar *et al.,* (2014) described that the suspension of 72h old *Xoo* isolates when inoculated in 45 days old rice plants by the clip-inoculation method developed BLB symptoms relatively in less time than pin prick method of inoculation.

## Phage isolation and characterization

Floodwater samples and BLB infected leaf samples were collected from paddy fields in 12 various locations of Madurai district and were analysed for the presence of *Xoo* phages. Two *Xoo* (*Xoo*M1 and *Xoo* A1) strains were used for the initial isolation. From these 12 samples, five phages were recovered and differentiated on the basis of plaque diameter (Table 3; Plate 3). *Xoo* Phage isolates *Xoo* Bp (MDU1), *Xoo* Bp (MDU3) and *Xoo* Bp S1 produced the clear plaques with diameter of 2.0, 1.8 and 2.4mm respectively. *Xoo* Phage isolates *Xoo* Bp (MDU2) and *Xoo* Bp M1 produced the clear and turbid plaques with diameter of 1.8 and 2.2 (Table 4; Plate 5).

The variation occurred in the plaque size might be due to the lytic effect of phages on *Xoo*. In concurrence with the findings an extensive research on this area was carried out by Balogh (2006) and Chae *et al.,* (2014).

In the present study the relative plaque forming efficiency of five isolates of phages was compared. The phage, *Xoo* Bp (MDU1) produced significantly higher number of plaques than the other four phages with the number of plaques formed as 138.45 (Table 4; Plate 5). Number of plaques varied to all isolates of phages due to the various lysis speed of phages. Similar finding on assessment of the lytic activity of phages on *Xoo* was reported by *Chae et al.,* (2014).

The effect of phage isolates against the virulent isolate *Xoo* M1 *in vitro* was assessed and the results revealed that the number of viable bacteria gradually decreased from $2.5\times10^5$CFU/ml at the start of the incubation to $0.13\times10^5$ CFU/ml after 120 min of incubation with *Xoo* Bp (MDU1), which recorded 94.8 percent reduction over control (Table 5; Plate 6). From the results it is observed that phage isoalte *Xoo* Bp (MDU1) was highly effective against *Xoo*M1 *in vitro* and preserved for further studies.

Watanabe *et al., (*2007) reported the similar finding in citrus, where phage treated bacterial culture of *Xanthomonas axonopodis* pv. *citri* had lower viable bacterial count than untreated bacteria.

In the present study an effective isolate of phage *Xoo* Bp (MDU1) was examined using a Transmission Electron Microscope (TEM) under 20 nm and 50 nm magnification. The size of the head of the phage isolate *Xoo* Bp (MDU1) was 61.8 x 55.7 nm and 95.9 nm was the length of tail.

Chae *et al.,* (2014) carried out the TEM study and recorded the size of Myoviridae bacteriophage had hexagonal out line 56.7 nm length and 60.8 nm widths.

The head of Siphoviridae was also hexagonal with width 53.5 and length 57.0 nm. The tail was 85 and 145 nm in length for the Myoviridae and Siphoviridae phages.

**Efficacy of various protective formulations of phage Xoo Bp MDU1 against the virulent isolate of *Xoo* M1 under artificially inoculated condition**

Phage Xoo Bp MDU1 was formulated in different protectants *viz.,* pregelatinised corn flour (PCF – 0.5% and 0.25%), Skim milk (0.05% and 0.75%), Sucrose (0.5%), Gluten (1%), Gelatin (0.5%). Likewise 11 formulations were prepared in combinations of the protectants and tested for their effect on BLB disease management. Among these 11 formulations, phage titer was found to be highest in T5 (skim milk (0.5%) + sucrose (0.5%) formulation) and the phage titre was 9.85 $x10^{10}$/ml. The skim milk (0.5%) + sucrose (0.5%) formulation recorded the lowest PDI of 33.58 and it was followed by skim milk (0.5%) with a PDI of 38.37. Chemical method of control (T 14) recorded the lowest PDI of 22.50 and sterile water control recorded the highest PDI of 56.66 (Table 6; Fig 1).

Results clearly depicted that the phage formulated with skim milk and sucrose expressed good reduction of BLB intensity with 40.73 per cent reduction over control. This might be by prolonging the retention of phages on paddy thereby phage could get an access to the pathogen, which resulted in better BLB management. Thus the variation in protectant activity of various carriers attributed the difference in BLB management ability of phage.

Balogh, 2002 reported in his experiment twice weekly applications of non-formulated phage did not reduce the bacterial spot disease severity significantly. The use of protective formulations was necessary to achieve significant control of the disease. The concern about environment-friendly sustainable agriculture and the rise of organic production necessitates improvements in biological disease control methods, including the use of bacteriophages against bacterial plant pathogens.

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