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Screening and formulation of novel carriers for *Xanthomonas* bacteriophage to control bacterial leaf blight disease

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ABSTRACT

Aims: This study was aimed to evaluate the potential of several carriers to formulate the phages and retain their activity under various pH and temperature conditions.
Methodology and results: The skim milk, rice flour, corn flour and CalnuXan (calcium and magnesium) as carriers to formulate the isolated phage to maintain its activity under extreme pH and temperature conditions. Two phages formulated with carriers retained their viability at pH 5, pH 7 and pH 9 compared to that of the unformulated phages. Besides, the formulated phages also retained a high titre compared to the unformulated phages when they were exposed to 37 °C and 45 °C. Based on the *in vitro* study of the formulation, it was applied in the glass house. The plant height, leaf chlorophyll and disease scoring were recorded and analyzed. In the glass house, the rice plant treated with formulated phages showed higher plant height and chlorophyll content than those treated with unformulated or untreated phages. Nonetheless, both formulated and unformulated protected the rice plant, which showed lower disease severity than the untreated group.
Conclusion, significance and impact of study: Phage therapy has been used for treating plant diseases caused by pathogenic bacteria. Despite their effectiveness in killing the pathogen *in vitro*, the results were not reproducible in the field. Bacteriophages (phages) are sensitive to environmental factors and infection efficiency was dropped when exposed to harmful environments. However, this study successfully formulated two novels *Xanthomonas* phages, as biocontrol agents against bacterial leaf blight (BLB) disease in rice.

Keywords: Antimicrobial, bacterial leaf blight, formulation, paddy, *Xanthomonas* phage

INTRODUCTION

Bacterial leaf blight (BLB) disease is one of the diseases in paddy that is caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), a pathogenic bacterium causing BLB, possesses a round-ended, rod-shaped cell. The length of individual cells varies from about 0.7 µm to 2.0 µm and width from 0.4 µm to 0.7 µm, respectively (Niño-Liu *et al*., 2006). Under the favourable condition, BLB spreads uncontrollably and causes massive destruction to the paddy leading to considerable rice losses and production (Swings *et al*., 1990; Arshad *et al*., 2015; Patil *et al*., 2017). Recently, bacteriophage (Phage), a virus that infects bacteria, has been reported as an alternative for treating plant diseases caused by pathogenic bacteria (Bae *et al*., 2012; Doss *et al*., 2017). Despite promising results *in vitro*, the effectiveness of the treatment diminished under high temperature, acidic or alkaline pH, sunlight and rain leaching (Ignoffo and Garcia, 1992). Therefore, the development of a protective formulation with phage is necessary for field application.

Formulation technologies were used to protect pesticide or biological agents from withstanding challenging environmental factors to enhance the efficiency of disease control. These formulations include solids, liquids and slurry formulations. Among these, the

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liquid formulation is preferable as the formulated products could be sprayed over a wide area of above-ground plants (Jambhulkar *et al*., 2016). In addition, polymer or lipid formulation of the phage, such as dried skimmed milk, formed a lipid coating surrounding the phages protecting them in the environment (Murthy and Engelhardt, 2008). The carrier substrate in the formulation is the most critical element as it helps in maintaining high numbers of the biocontrol agent yet does not demonstrate biocontrol potential (Keswani *et al*., 2016). In the early study, Balogh (2002) used skim milk, casecrete, sucrose and pregelatinised corn flour for the formulation of *X. camperstris* pv. *vesicatoria* phages. Skim milk and corn flour have been shown to be stable carriers formulate the phage (Iriarte *et al.*, 2007; Tewfike and Desoky, 2015; Orynbayev *et al.*, 2020). Little study has been done on rice flour and CalnuXan as a formulation for phages. This study aimed to formulate *Xoo* Nϕ-1 and Nϕ-3 phages using skim milk, rice flour, corn flour and CalnuXan (calcium and magnesium) as a carrier and to investigate their effects on the phage activities in the *in vivo* study.

MATERIALS AND METHODS

Recovering of bacterial pathogens and bacteriophage

Xanthomonas oryzae pv. *oryzae* (Code: UPMC 619) strain was obtained from Microbial Culture Collection Unit (UNiCC), Institute of Bioscience, Universiti Putra Malaysia. The *Xoo* was recovered and cultured on nutrient agar (NA) plates and nutrient broth (NB, Merck). The freeze-dried culture was incubated at room temperature for a few minutes and then transferred into NB, followed by incubation with 200 rpm shaking at 37 °C. Two isolated phages (Nϕ-1 and Nϕ-3) from termites, as described previously in Liu *et al*. (2021) were used.

Sample preparation

Phage formulations were freshly prepared by mixing the phage lysate (10^8 pfu/mL) with different concentrations of sterile carriers (Table 1) prior to the experiment.

Table 1: Formulation of carriers in this study.

Name of carriers	Formulation
Skim milk power (Brand: Oxoid)	0.5% (w/v)
	0.75% (w/v)
	1% (w/v)
CalnuXan (Brand: Evergreen)	0.5% (w/v)
	0.75% (w/v)
	1% (w/v)
Rice flour (Brand: Erawan)	0.5% (w/v)
	0.75% (w/v)
	1% (w/v)
Corn flour (Brand: Cap Bintang)	0.5% (w/v)
	0.75% (w/v)
	1% (w/v)

Effect of pH on stability of formulated *Xoo* phages

The formulated phages Nϕ-1 and Nϕ-3 were tested for their stability in sterile sodium magnesium (SM) buffer (100 mM of NaCl, 8 mM of $MgSO_4.7H_2O$, 50 mM of Tris-HCl, pH 7) at various pH. Briefly, the tested phages lysate (10^8 pfu/mL) were diluted in SM buffer at different pH (5.0, 7.0 and 9.0), followed by the addition of equal volumes of carriers at different ratios (Table 1). The formulated phages were incubated at 37 °C for 1, 12 and 24 h. Each treated formulation was mixed with the host bacterium at the mid-exponential phase (10^8 cfu/mL) followed by plaque assay using the double-layer agar technique. The titre of phages was calculated as a plaque-forming unit (pfu/mL). The experiment was done in triplicates.

Effect of temperature on the stability of formulated *Xoo* phages

The stability of formulated phages in sterile SM buffer under various temperatures was evaluated. The phage lysates Nϕ-1 and Nϕ-3 (10^8 pfu/mL) formulations were prepared as described in Table 1 and were incubated at different temperatures (room temperature at 25 °C, 37 °C and 45 °C) for designated periods (1 day, 7 days and 14 days). After incubation, a plaque assay was performed to determine the phage titre. The experiment was done in triplicates.

Preparation of bacterial host and formulation bacteriophage for field trial

Xanthomonas oryzae pv. *oryzae* (*Xoo*) strain was prepared for challenging study in the greenhouse. Approximately 100 mL of overnight *Xoo* culture was added to 1 litre of fresh NB and incubated at 37 °C on a 200 rpm shaker until the absorbance of bacterial suspension reached OD_{600nm} of 0.6-0.8 (10^{8-9} cfu/mL). Phage Nϕ-1 and Nϕ-3 (10^8 pfu/mL) were mixed with different type of carriers at a ratio of 1:1. The phage formulation was then delivered onto the foliage of rice plants with hand-held sprayers.

Glasshouse study

Rice seedlings (MR219) were grown in 16 × 16 inches and 0.15 mm thickness poly bags in 8 × 8 m glasshouse. Each treatment consisted of 3 poly bags of rice seedlings and the inter-row spacing was 60 cm. The plants were grown to the 4-5 leaf stage in the glasshouse. Leaves from 50-day-old were brushed with a toothbrush to injure the surface of leaves (at the tillering stage) of rice cultivars and subsequently inoculated with *Xoo* culture (10^9 cfu/mL) using a hand-held plastic sprayer until it was completely wet. Infected rice plants were grown under normal glasshouse conditions.

According to Figures 4 and 5, Nϕ-1 phage was selected for the glasshouse study due to the keep high titre under the survival temperature compared with Nϕ-3 phage. The suspension of formulated phages

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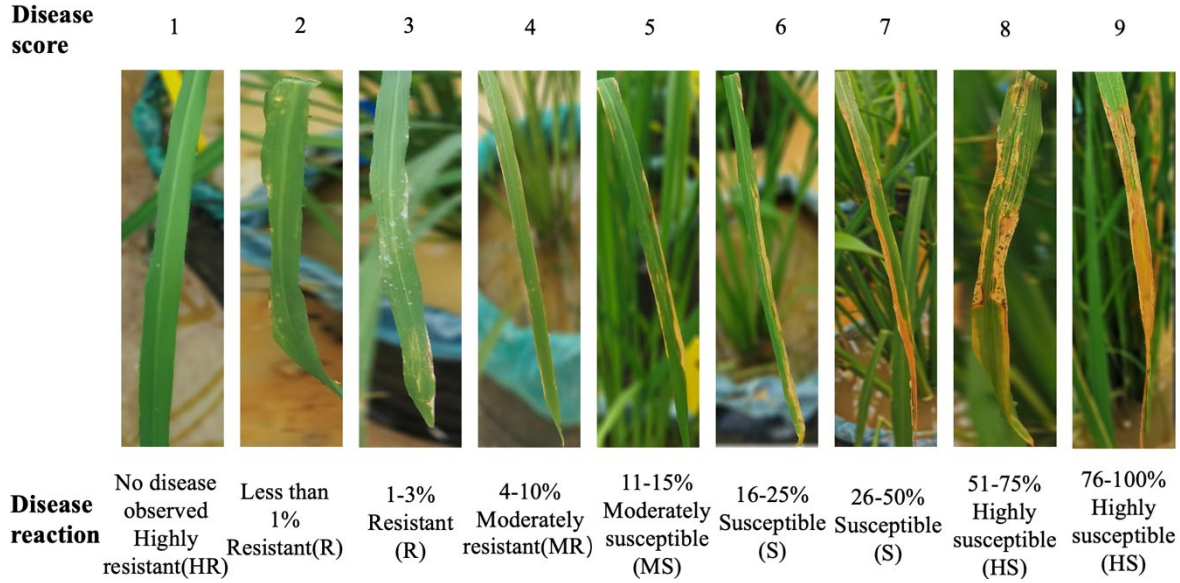

Figure 1: Disease severity (score) diagram to estimate symptoms of bacterial blight disease on rice.

Table 2: Treatment used in the glasshouse study.

Name	Information
T1	0.5% (w/v) Skim milk powder + phage (Ratio 1:1)
T2	0.5% (w/v) Rice flour + phage (Ratio 1:1)
T3	0.5% (w/v) Corn flour + phage (Ratio 1:1)
T4	Positive Control (Unchallenged, Untreated)
T5	Nϕ-1 Phage (Challenged with *Xoo*, Treated with phage only)
T6	Negative Control (Challenged with *Xoo*, Untreated)

Table 3: Disease severity scale for evaluation of bacterial leaf blight of rice in the field (IRRI, 2013).

Disease score	Lesion area (%)	Disease reaction
1	0	Highly resistant (HR)
2	<1	Resistant (R)
3	1-3	Resistant (R)
4	4-10	Moderately resistant (MR)
5	11-15	Moderately susceptible (MS)
6	16-25	Susceptible (S)
7	26-50	Susceptible (S)
8	51-75	Highly susceptible (HS)
9	76-100	Highly susceptible (HS)

(approximately 10^8 pfu/mL) was used as a biocontrol agent. All treatments were applied weekly in the evening, a total of seven times for the whole study and disease symptoms were observed every 7 days post-treatment. Control plants were treated with tap water and carriers (skim milk, rice flour and corn flour). The treatment used in the study is shown in Table 2.

Data collection

Plant height, leaf chlorophyll and disease scoring were collected from the treatment for 0, 7, 14, 21, 28, 35 and 42 days post-treatment. Plant height was measured by a straightedge and leaf chlorophyll was measured by Chlorophyll Meter SPAD-502Plus (Konica Minolta, Germany). The disease severity was scored according to a recommendation from International Rice Research Institute (IRRI, 2013), as shown in Figure 1 and Table 3.

Experimental design and statistical analysis

Each treatment consists of three replicates. The plots were set up in a completely randomised design (CRD). Data collected were subjected to an analysis of variance (ANOVA) to determine significant differences between each treatment by Duncan's test at $p<0.05$.

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RESULTS

Phage titration of formulated *Xoo* phages

The *Xoo* phages (Nϕ-1 and Nϕ-3) were formulated with different carries, i.e., skim milk, CalnuXan, rice flour and corn flour at a ratio of 1:1. Plaques were present in all the formulated *Xoo* phages (Table 4) proving that the formulation did not affect the infectivity of the phage against the bacterial host and could be used for subsequent studies.

Effect of pH on stability of formulated *Xoo* phage

The effect of pH on formulated *Xoo* phage infectivity was investigated by exposing the selected phages to a range of acidic (pH 5), neutral (pH 7) and alkaline (pH 9) environments for 1 h, 12 h and 24 h. The titres of the formulated phages after treatment are shown in Figure 2 and Figure 3. All formulated phages retained their activity at pH 5, pH 7 and pH 9. Among these, pH 7 remained the optimal pH compared to other pH treatments, showing a higher titre by approximately 1-2 log10 pfu/mL following 24 h exposure to 37 °C.

The phages stability under pH 5, 7 and 9 varied among different formulated phages. Nϕ-1 and Nϕ-3 phages with skim milk, rice flour and corn flour carriers were more stable than phages formulated with CalnuXan at 37 °C incubation in 1-24 h, with approximately 1-3 log10 reduction in phage titres. There is no significant difference in phage stability and phages titre in the different concentrations of all carriers. As the incubation time increased, the unformulated phage titre dropped approximately 1-3 log10 in pH 5 and pH 9, indicating that these phages were not stable in acidic and basic conditions without the presence of carriers. When the Nϕ-1 and Nϕ-3 phages were formulated with skim milk and rice flour, they maintained a high titre of phage in these pH conditions. On the other hand, corn flour also retained high infectivity during the first hours but was reduced by a log10 in titre at 12 and 24 h. In contrast, the carriers CalnuXan showed low phages titres in the acidic and basic conditions. This group of formulated phages showed similar titres or less similar to that of the unformulated phages control group suggesting that CalnuXan carrier was ineffective in protecting the phages in an acidic or basic environment. The results of this study suggested that skim milk, rice flour and corn flour are better carriers to protect phages and shall be used to formulate phages.

Effect of temperature on the stability of formulated *Xoo* phage

Different temperatures' effect on formulated phage stability was determined in sterile SM buffer adjusted to pH 7.5. Formulated and unformulated Nϕ-1 and Nϕ-3 phages showed a log reduction in phage titre when exposed to 45 °C after 7 days (Figure 4 and Figure 5). When they were exposed to the same temperature for 14 days, no plaque formed, suggesting that no infectious formulated phages could be recovered. In contrast, only a little or no reduction in virus titre when these phages were exposed to the same temperature for a day. These results suggested that the phages could retain their infectivity even though they have been exposed to temperature as high as 45 °C during transportation. Prolonged storage of these phages in a room at 45 °C will render them ineffective as biological control agents for plant diseases.

On the other hand, when the formulated and unformulated phages were exposed to 37 °C for 14 days, a reduction of 1-2 log10 was observed, generally, in different phages and formulations. However, no reduction or only a slight reduction (less than a log10) in virus titre was observed when these phages were exposed to 37 °C for 1 or 7 days, respectively. No reduction in virus titre was observed for all the formulated phages when they were kept at room temperature for up to 14 days, but a slight reduction (~1 log) for unformulated phages in the same condition.

Plant physiology (height and chlorophyll) of rice plant

Upon infection of *Xoo* in the rice plant during the tillering stage, the growth of the plant and the chlorophyll content of the leaves would be affected. Figure 6 shows the rice plant with BLB symptoms with and without treatment. In the presence of treatment, the effect of the infection was substantially reduced. The effects of the treatments were measured quantitatively and statistically analysed.

All treated and untreated rice plants showed no significant difference in plant height on days 0, 7, 14 and 21 (Figure 7), suggesting that BLB disease does not affect the rice plant height during the initial stage of rice plants. However, the height of rice plants treated with formulated phages showed significant differences compared to that of the untreated group on days 28, 35 and 42 (Figure 7). The T1-T3 (treatment group) showed higher values than the T5 (Nϕ-1 *Xoo* phage only) at 28, 35 and 42 days (Figure 7). However, T5 showed higher values than T6 (untreated control). This suggested that the formulated phages have the potential to inhibit BLB development and it enhanced plant growth in terms of plant height at the vegetative stage. Nevertheless, there were no significant differences among the formulated treatments T1-T3. These results indicated that the different types of carriers have the same capacity to formulate phages under the glasshouse condition.

The chlorophyll content of rice plants is a good indicator of leaf greenness, leaf photosynthesis and yield potential. In this study, a substantial reduction in chlorophyll contents for those untreated rice plants was detected from day 14 onwards in all the groups (Figure 8). The unformulated Nϕ-1 *Xoo* phage (T5) showed significantly lower chlorophyll content than the formulated control (T1-T3) but significantly higher chlorophyll content compared to those of the untreated control.

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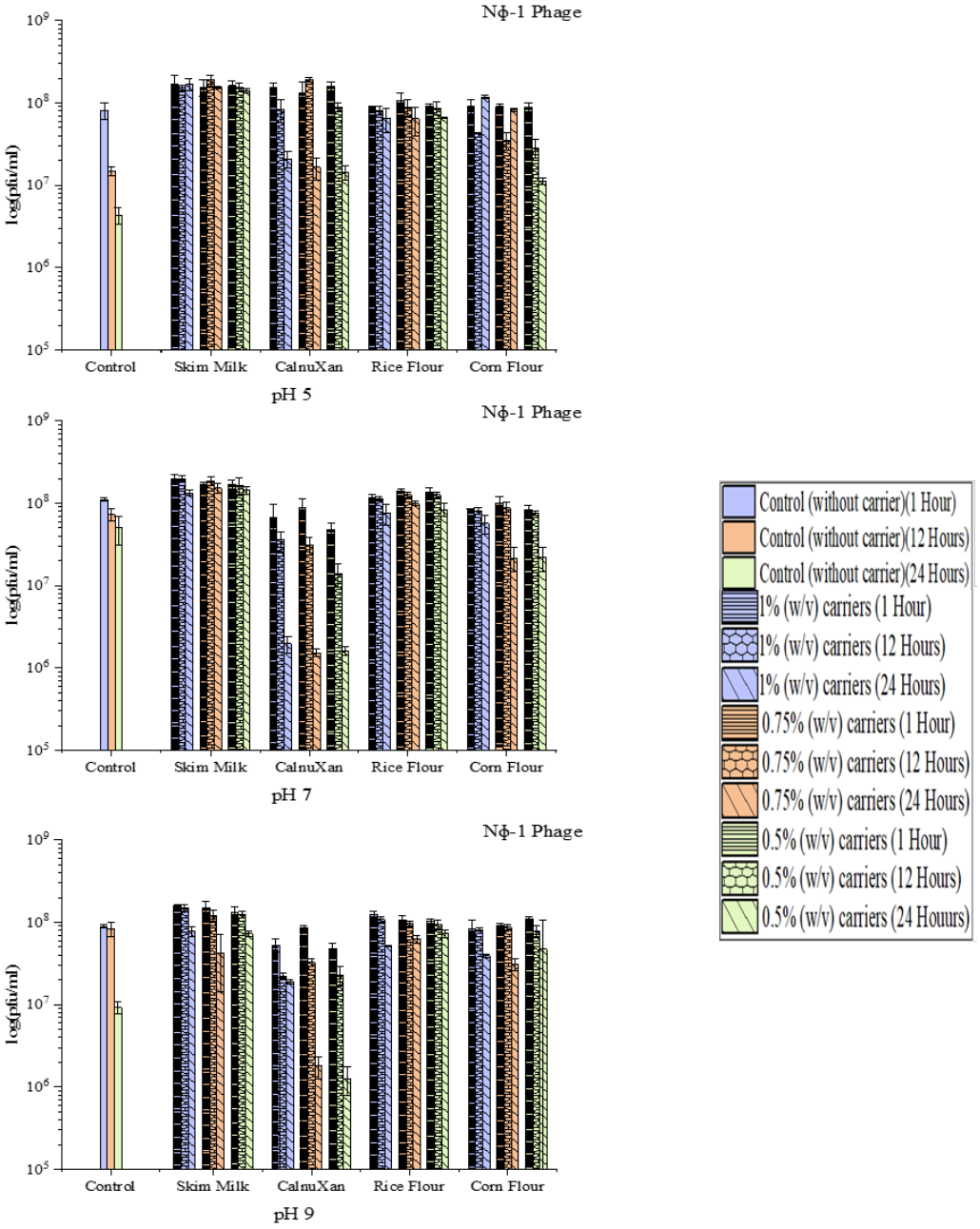

Figure 2: Stability of formulated *Xoo* phages Nϕ-1 in different pH conditions. The phages were formulated with skim milk, CalnuXan, rice flour and corn flour at three different concentrations of carriers (0.5% w/v, 0.75% w/v and 1% w/v) and were incubated at pH 5, pH 7 and pH 9 buffers for 1 h, 12 h and 24 h at 37 °C. The non-formulated phage was used as a control. The results are the mean values obtained from three independent replicates and the error bars represent the standard deviation.

Table 4: *Xoo* phages (Nϕ-1 and Nϕ-3) encapsulated with skim milk, CalnuXan, rice flour and corn flour.

Phage	Skim milk	CanluXan	Rice flour	Corn flour
Nϕ-1	+	+	+	+
Nϕ-3	+	+	+	+

+: Plaque formation detected; -: No plaque formation detected.

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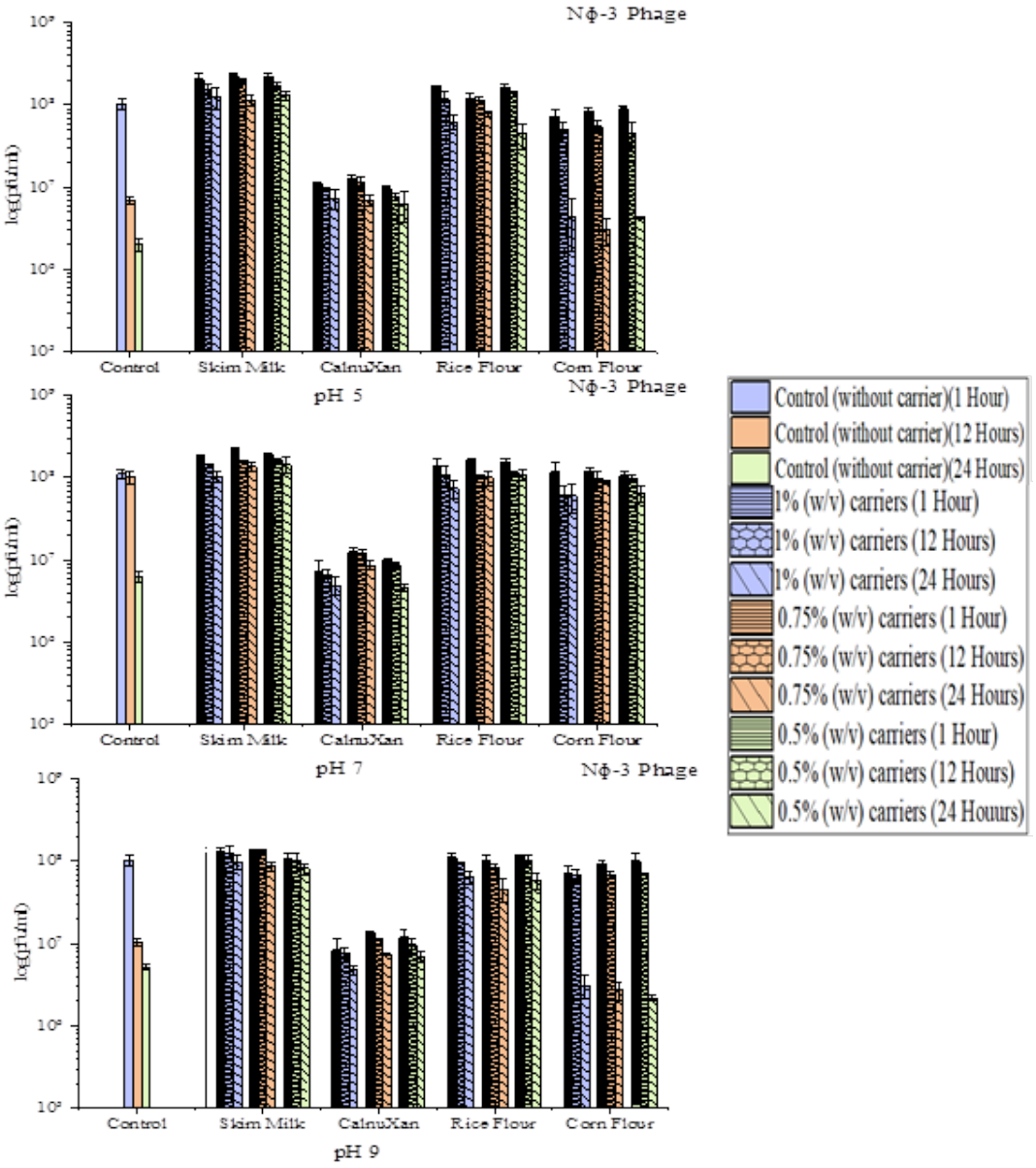

Figure 3: Stability of formulated *Xoo* phages Nϕ-3 in different pH conditions. The phages were formulated with skim milk, CalnuXan, rice flour and corn flour at three different concentrations of carriers (0.5% w/v, 0.75% w/v and 1% w/v) and were incubated at pH 5, pH 7 and pH 9 buffers for 1 h, 12 h and 24 h at 37 °C. The non-formulated phage was used as a control. The results are the mean values obtained from three independent replicates and the error bars represent the standard deviation.

Disease progression of *Xanthomonas oryzae* pv. *oryzae* in rice plant

The disease severity index on every data collection day gave an overview of the disease progression for the rice plant with and without treatment. It could be seen from Figure 9 that the rice plant has relatively low disease severity (below 30%) for all the treatment groups with or without formulation at day 14 compared to those of the untreated rice plant that showed above 60% severity. The severity of these untreated plants reached maximum severity as early as day 21 and almost plateaued after that. However, those treated plants increased gradually to the highest severity on day 42. There were no significant differences among the formulations in all the treatment groups. Nevertheless, a slightly significant difference was observed between formulated and unformulated phages at a particular time point.

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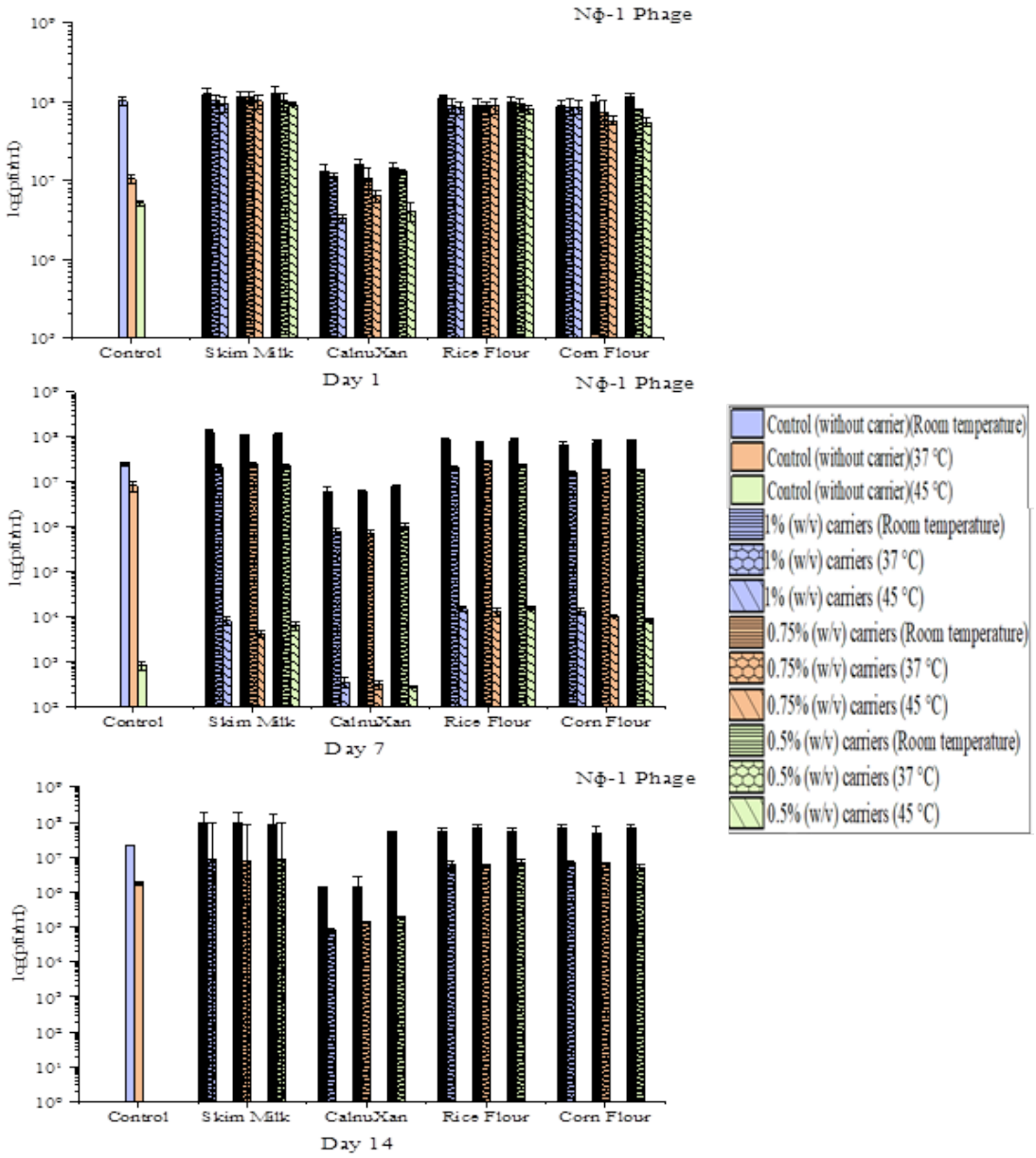

Figure 4: Effect of temperature on the survival of unformulated and formulated *Xoo* phages Nϕ-1. The phages were maintained at room temperature, 37 °C and 45 °C for 1 day, 7 days and 14 days. The formulations (skim milk, CalnuXan, rice flour and corn flour) at different concentrations [0.5%, 0.75% and 1% (w/v)] were used. The results are the mean values obtained from three independent replicates and the error bars represent the standard deviation.

DISCUSSION

Environmental factors such as pH is an essential factor in determining the success of phage therapy. The pH affects phage attachment, infectivity, intracellular replication and new progeny amplification (Pirisi, 2000; Leverentz *et al.*, 2001; Leverentz *et al.*, 2004; Jończyk *et al.*, 2011). Under unfavourable pH conditions, the phages-host interaction, particularly the activities of lysozyme and other phage capsid proteins, prevents the phages from attaching to the receptors on the host cell (Pirisi, 2000; Leverentz *et al.*, 2004). Therefore, it is essential to determine the pH stability of the formulated phages before they can be used as a biocontrol agent for rice pathogens. In this study, two phages were formulated with different carriers (skim milk, CalnuXan, rice flour and corn flour), followed by exposure to a range of pH values (pH 5, pH 7 and pH 9). In general, all formulated and unformulated phages were found to retain their infectivity under pH 7 (Figure 2 and Figure 3). In line with this study, most of the previous studies

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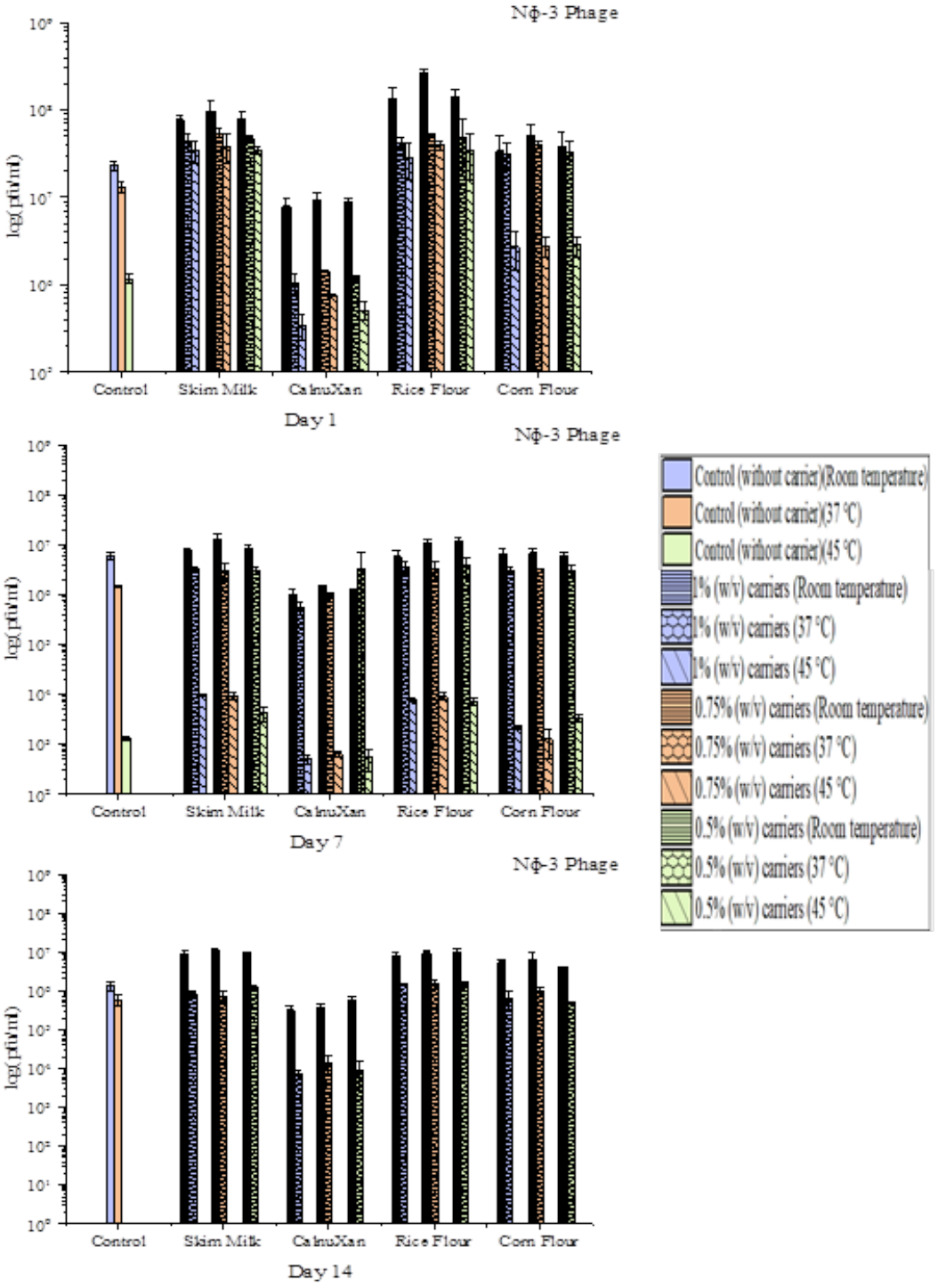

Figure 5: Effect of temperature on the survival of unformulated and formulated *Xoo* phages Nϕ-3. The phages were maintained at room temperature, 37 °C and 45 °C for 1 day, 7 days and 14 days. The formulations (skim milk, CaluXan, rice flour and corn flour) at different concentrations [0.5%, 0.75% and 1% (w/v)] were used. The results are the mean values obtained from three independent replicates and the error bars represent the standard deviation.

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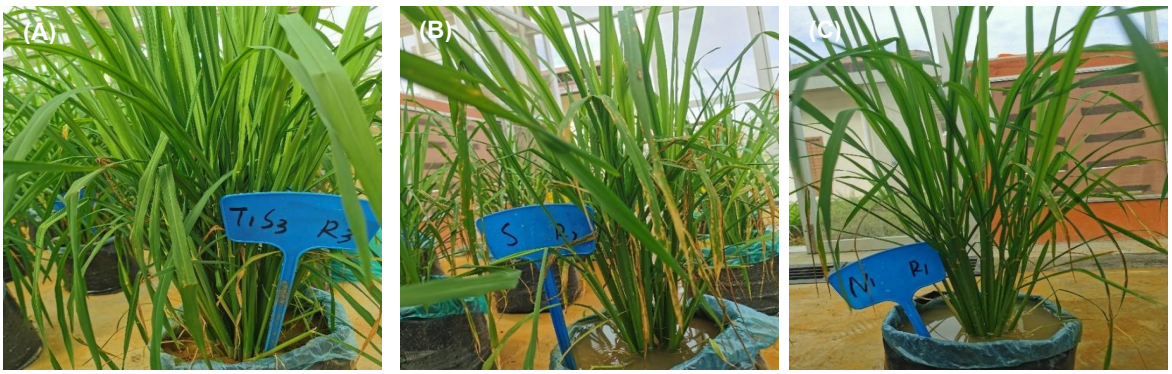

Figure 6: Rice plant with characteristic bacterial leaf blight disease symptoms on 28 days after post-treatment. (A) T1: Nϕ-1 Phage formulation with skim milk (challenged, treated with phage 10^8 pfu/mL); (B) T6: Negative control (challenged, untreated); (C) T5: Nϕ-1 Phage (challenged, treated with phage 10^8 pfu/mL).

reported that various phages such as T7, T4 and T2 are stable at pH 6.0-8.0 and retain their infectivity in that condition (Kerby *et al*., 1949, Klak *et al*., 2010; Jończyk *et al*., 2011; Elhalag *et al*., 2018).

In acidic pH, many viruses are unstable in the absence of formulation and lose a certain percentage, some up to 100% of their infectivity after storage (Kerby *et al*., 1949; Jończyk *et al*., 2011, Endersen *et al.*, 2017). Similarly, in alkaline conditions, some phages such as T7 were reported to retain only 30% of their infectivity after incubation for 14 days at pH 9 and even lost their activity completely at pH more than 10 after 24 h (Jończyk *et al*., 2011). In the meantime, our results showed that the Nϕ-1 and Nϕ-3 *Xoo* phages without formulation were also sensitive to pH 5 and reduced phage titre was observed. This is proven that phages formulation is crucial to retaining the infectivity of the phages. Phages formulated with skim milk and rice flour are believed to protect the phages at pH 5, which helped maintain the phage infectivity and hence comparable phage titre to that of the neutral pH. These results are in agreement with the observation by Abdelsattar *et al.* (2019) that explained that bead-formulation provides protection for phage against acid stress with approximately one log10 PFU reduction observed at pH 2 compared to complete inactivation of the free phage. In addition, microencapsulated *Escherichia coli* O157:H7 phage preserves higher lytic ability than the unencapsulated phage under different pH range values (pH 3-7) (Ramirez *et al*., 2018). These findings proved that the phage formulation using various carriers such as skim milk, rice flour and corn flour could protect phages under high and low pH conditions. Proteins added in the phage formulations were to help preserve the activity and possibly generate the synergistic therapeutic effect. However, it is inconclusive to elucidate the effects of protein on phage stability since only casein and lactoferrin have been investigated in a few studies. Casein and lactoferrin abundantly present in milk and milk secretions have been approved in oral tablets by FDA. Both were proved to be effective in retaining the stability of phages during processing or storage when incorporated into phage formulations (Zhang *et al.*, 2020). For example, some scientists found that the incorporation of phages into microparticles composed of casein sodium salt, trehalose and leucine could reduce the processing loss during low-temperature spray-drying (Matinkhoo *et al.*, 2011). Further, the encapsulation resulted in less than 0.15 log titer loss after 90 days of refrigerated storage. In another study, a lactose/lactoferrin (60/40, w/w) matrix was employed to encapsulate phages, which hardly led to the loss of viability during lyophilization and no significant loss in titer was observed after 90 days of storage at 4 and 22 °C (Golshahi *et al.*, 2011). Further studies are warranted using these and other protein excipients to formulate phage (Zhang *et al*., 2020).

The formulation plays a vital role in determining the infectivity of the virus. However, no single formulation is perfect for all phages to retain their infectivity in various environments. The four carriers used in this study showed variable results for different phages. Generally, CalnuXan can be considered the least effective formulation to protect the phages against various temperatures regardless of the total number of days of incubation. The main component of CalnuXan is calcium and magnesium. Chow *et al*. (1971) and Jończyk-Matysiak *et al.* (2019) mentioned that Xp12 phage (Siphoviridae) was unstable in different ionic environments, such as Ca^{2+}, Mg^{2+} and Fe^{2+} at room temperature, which is comparable with our result. In contrast, adding magnesium ions to *Escherichia coli* phages can retain the phage titre after incubating at 30 °C for one month (Bourdin *et al*., 2014). However, in this study, CalnuXan could not retain the titre of the phage. This might be because of the influences of other factors, which unstabilised the phage virions.

In addition to pH, temperature is another critical factor influencing phages' lytic activity during the attachment, penetration and new progeny produced in their host cells (Nasser and Oman, 1999; Olson *et al*., 2004; Jończyk *et al.*, 2011; Pinheiro *et al*., 2019). This study showed that all formulated or unformulated phages lost the virus infectivity when they were incubated at 45 °C for 14 days. This result was similar to that reported by Brown *et al*. (2016), who showed a total loss of lytic capacity of the

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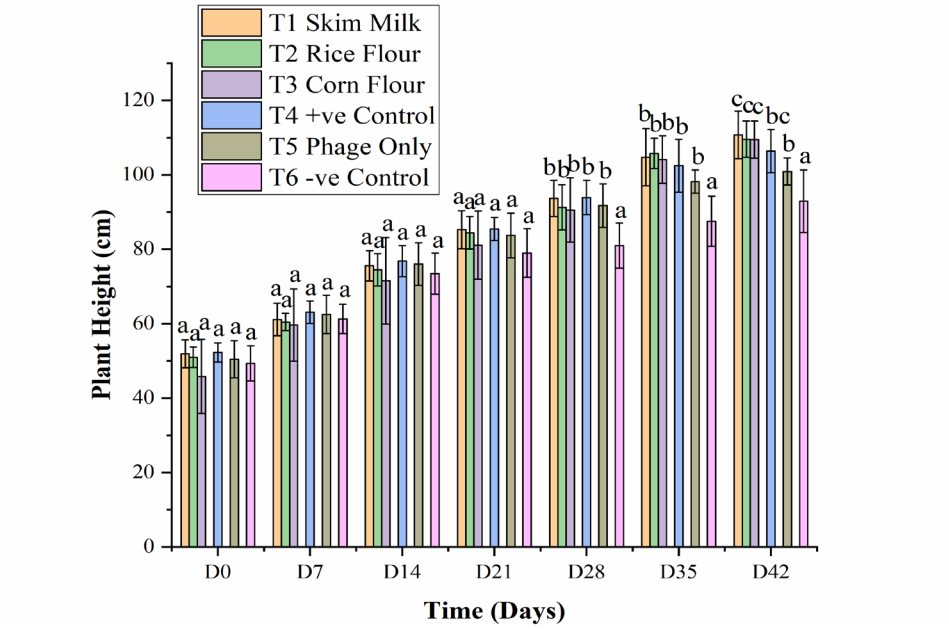

Figure 7: The average plant height of rice plants treated. Measurement was recorded on days 0, 7, 14, 21, 28, 35 and 42. The positive control represents unchallenged and untreated treatment, while the negative control represents challenged *Xoo* with untreated. The Standard measuring ruler was used to measure the plant width and height. Each plant was measured 3 times on the data collection day. Mean followed by different letters are significantly different at $p<0.05$ level according to Duncan's one-way ANOVA. The same letter above the bar represented no significant difference between treatments.

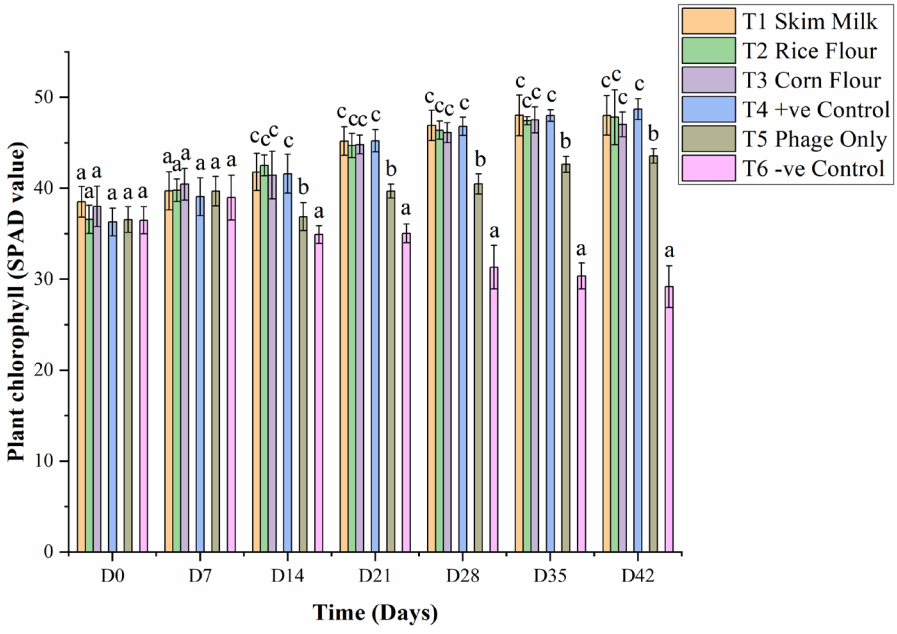

Figure 8: The average plant chlorophyll of rice plants treated. Measurement was recorded on days 0, 7, 14, 21, 28, 35 and 42. The positive control represents unchallenged and untreated treatment, while the negative control represents challenged *Xoo* with untreated. The Standard measuring ruler was used to measure the plant width and height. Each plant was measured 3 times on the data collection day. Mean followed by different letters are significantly different at $p<0.05$ level according to Duncan's one-way ANOVA. The same letter above the bar represented no significant difference between treatments.

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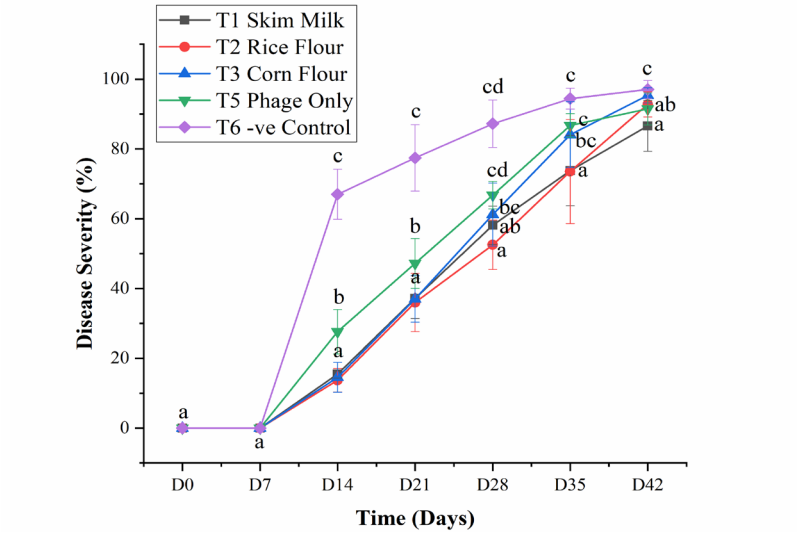

Figure 9: The average plant disease severity percentage of rice plants infected with *Xanthomonas oryzae* pv. *oryzae* in the glass house with and without treatment. The positive control represents unchallenged and untreated treatment, while the negative control represents challenged *Xoo* with untreated. All the data was recorded on days 14, 21, 28, 35 and 42 post-infection. Mean followed by different letters are significantly different at $p<0.05$ level according to Duncan's one-way ANOVA. The same letters above the bar represent no significant difference between treatments.

phages formulated with cream after being stored at 45 °C for 14 days. Moreover, the four lytic phages against *Salmonella newport* showed a significant reduction in phage titre when the phages were kept at 45 °C and 55 °C for 2 weeks (El-Dougdoug *et al.*, 2019). However, only 1~2 log reductions were observed when the phages with the formulation were incubated at such temperatures for a day. Yin *et al.* (2019) showed that φXWY0014 and φXWY0026 phage titre reduced by 2~3 log10, whereas φXWY0013 titre reduced by 1 log10 after an hour of exposure to 60 °C. Besides, Endersen *et al.* (2017) showed that phage IeB, IeE and IeN lost their activity following 1 h exposure to 60 °C.

In this study, our results showed that the formulated phage could increase the longevity of phages under room temperature. Abo-elmaaty *et al.* (2016) showed that unformulated phage populations were quickly reduced at room temperature after 36 h and 48 h whilst formulated phages decreased in population only after two days. Moreover, Iriarte *et al.* (2007) demonstrated that ϕXV3-16 and ϕXacm 2004-16, formulated with skim milk added with sucrose, survived for 10 days at 28 and 32 °C. The same trend was observed for formulated phage of this study. All formulated phages with skim milk, corn flour and rice flour showed a higher titre than free phages at room temperature for 1 day, 7 days and 14 days. However, the CalnuXan showed no protective effect for phages.

The rice plant physiology (height and chlorophyll) was evaluated. Above-ground-biomass (AGB) is a crucial indicator for estimating the productivity of many crops within different ecosystems (Liu *et al.*, 2019). So, it is essential to evaluate the AGB during the rice growth stage. In this study, the plant height of treated plants (T1-T3) was higher and broader than those of the untreated plants (T6) at 28, 35 and 42 days after treatment in the glasshouse. This is due to the presence of phage to inhibit disease development. It was conducted similarly to the study by Ranjani *et al.* (2018), where φXOF4 phage (Siphoviridae) was used to inhibit the BLB disease development.

Xoo infects the rice plant maximally at a tillering stage affecting the rice plant physiology such as height and chlorophyll and consequently grain yield loss (Yasmin *et al.*, 2017). Hence, the subsequent analyses focused on plant physiology and grain production with and without the treatment of phages. The morphology and physiological features of the rice plant below and above the ground surface are the main factors determining grain yield (Thakur *et al.*, 2011). Upon infection of *Xoo* on the tillers of the plant, a quick wilting of the entire plant occurred in 2-3 weeks (Niño-Liu *et al.*, 2006; Chukwu *et al.*, 2019; Thind, 2019). An example of the infected rice plant with BLB symptoms is shown in Figure 6B. According to Chukwu *et al.* (2019), the kresek phase of BLB is severe in tropical areas, such as Malaysia. This phase could be detected as yellowish and greyish with a roll and droop on infected leaves, which showed the same symptoms in our results. Then, the rice plant suffers tiller growth arrest and subsequently withers away with a stunted appearance (Niño-Liu *et al.*, 2006; Laha, 2017; Thind, 2019). In contrast, the treated plants showed healthy leaf blades (Figure 6A), which would improve the green plant biomass due to high nutrient uptake, such as nitrogen (Bianculli *et al.*, 2016; Liu *et al.*, 2019).

The formulated phages could control the BLB disease and improve the number of rice plants tiller. In numerous trials, using the unformulated phage to control the plant

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disease did not meet the objective. For example, McNeil *et al*. (2001) demonstrated that unformulated phages did not survive on walnut leaves in a greenhouse trial. Rapid degradation of phages limits its treatment's usefulness and causes the need for recurrent applications. Sunlight irradiation, temperature and pH affect both phage longevity on plant foliage and reduce the treatment efficacy (Ignoffo *et al*., 1997; Balogh, 2002; Iriarte *et al*., 2007; Jones *et al*., 2018). Hence, formulation to protect phages against these environmental factors is essential for prolonged viability of the phage to control BLB disease. Reduction in disease incidence is reflected in plant growth. Thakur *et al.* (2011) indicated that healthy plants in terms of culm height, tiller number per hill, tiller perimeter, leaf size and number, leaf area index (LAI) and open canopy structure would increase grain yield by 48%.

The chlorophyll was essential to the elevation of the crop of N-deficiency, leaf nitrogen content, biomass and yield in agriculture (Cerovic *et al.*, 2012; Liu *et al*., 2019). Moreover, Moharana and Dutta (2016) also described that chlorophyll and nitrogen content are vital to ing crop productivity and inquiring about crop stresses and nutritional state. It was shown by Zhao *et al.* (2019) that higher chlorophyll content and strong stay-green traits related to growth status and improvement of photosynthetic efficiency in rice plants.

The phyllosphere is a harsh environment and unformulated phages applied to aerial tissues degrade rapidly (McNeil *et al*., 2001; Balogh, 2002; Balogh *et al*., 2003). However, previous studies have shown that formulated phages can control plant diseases in the phyllosphere by enhancing phage longevity in-field practices (Balogh, 2002; Balogh *et al*., 2003; Obradovic *et al*., 2004). Results from this trial showed that all the carriers (skim milk, CalnuXan, rice flour and corn flour) could protect the phages from the harsh environment, retaining their capacity to control the BLB disease and increasing the chlorophyll content of rice leaves. Similar findings were reported by Balogh *et al*. (2003) that 0.75% powdered milk + 0.5% sucrose formulation protected the phage and increased the phage longevity on the leaves surface of tomato plants in greenhouse and field trials. In addition, phages formulated with skimmed milk or riboflavin also increased the phage titre 6.7 and 5.0 times, respectively, while increasing the phage's survival day to 5-8 days (Orynbayev *et al*., 2020). Tewfike and Desoky (2015) also reported that 0.5% corn flour and 0.5% skim milk formulated phage in the greenhouse and open field trials increased phage longevity on leaves surface for 20 to 50 h. Besides, extracted nature product formulations also increased the viability of phages two days after the application (Iriarte *et al*., 2007). It was shown that corn flour and skim milk could protect the phages to reside on plant foliage longer and subsequently protect the plant against diseases, increasing the leaves' chlorophyll content. The plant chlorophyll content, which could be determined by SPAD values, is closely related to plant health (Putri *et al*., 2016).

Phages are highly specific to the target bacteria and have enormous potential to be used as biocontrol agents (Adachi *et al.*, 2012). They have been successfully used to control several plant diseases to increase the plant's yield (Obradovic *et al*., 2004). In this study, the phages, either formulated or unformulated, successfully controlled the BLB disease resulting in an increase in the rice plant yield. BLB disease could cause a yield loss ranging from 20-30% and in some severe cases, the yield could be reduced up to 50% and cause economic loss in Malaysia (Mew *et al*., 1993; Kogeethavani *et al.*, 2021). Therefore, it is vital to control the disease to sustain rice production and feed the growing world population. In this study, the phage formulations increased the efficacy of phage treatments to control the disease and showed lower disease severity (%) compared to the untreated group and unformulated phages.

Many research groups have used skim milk to formulate phages to protect and enhance its efficacy in controlling the plant pathogen. Balogh *et al.* (2003) showed that skim milk formulated phages achieved the most significant reduction for bacterial spot disease on tomatoes, which was reduced by 79% and 45% in the first and second experiment, respectively. In addition, he also demonstrated that phage formulated with pregelatinised corn flour and casecrete enhanced the residual phage activity and disease control efficacy. On the other hand, Ibrahim *et al*. (2017) also showed that citrus canker disease is caused by *Xanthomonas citri* subsp. *crtri* (*Xcc*) treated with formulated phage showed lower disease incidence than the untreated control. Tewfike and Desoky (2015) also reported that formulated phages using corn flour and skim milk successfully controlled *Xanthomonas axonopodis* that caused bacterial spots reducing the disease severity to 20.5 and 18.3% in greenhouse conditions.

In addition to formulation, the virus titre might be one of the influencing factors affecting the disease control efficiency. Various virus titres (10^5-10^8 pfu/mL) were used in the treatment of *Xoo* in the study by Lang *et al.* (2007). Despite the reduction of disease severity observed in the phage-treated group, the insignificant difference among various titres used were reported. Nevertheless, Ranjani *et al.* (2018) demonstrated that *Xoo* inoculum at 1×10^7 cfu/mL caused 80% of disease manifestation of rice seed, affecting the seed germination but could be controlled successfully using phages at 1×10^8 pfu/mL (Ranjani *et al*., 2018) resulting in 0% disease incidence. In this study, the phage at 1×10^8 pfu/mL with or without formulation was used and showed a significant inhibitory effect on the disease on days 14, 21, 28 and 35 post-treatment (Figure 9). Timing of phages application influenced disease control's efficacy in several instances. In this study, treatment was carried out in the evening to prevent the possibility of exposure to environmental challenges such as UV and high temperature, which enable prolonged exposure of the bacteria to the phages. Iriarte *et al*. (2007) showed that formulated phages with skim milk and corn flour on tomato leaf surfaces persisted better when they were applied in the evening rather than in the morning. Obradovic *et al.* (2004) also indicated that evening applications of phage gave better treatment

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results than morning applications. Moreover, the phage population was preserved at relatively high levels during the evening and night (Jones *et al.*, 2018). Bigger coverage of the phage on the rice plant could also affect the disease control efficacy. Using foliar spraying, it was shown that phages effectively decreased disease incidence caused by *X. campestris* pv. *campestris* (Nagai *et al*., 2017), *Xanthomonas euvesicatoria* (Gašić *et al.*, 2018) and *P. carotovorum* subsp. *carotovorum* (Lim *et al*., 2013). Droplets of the phages from the hand-held spray covered the plants more effectively, resulting in better control of the BLB disease.

CONCLUSION

Phages formulated with skim milk, rice flour and corn flour were found to protect the phages against unfavourable conditions such as acidic or basic environment as well as high temperature for a duration of time. Nevertheless, prolonged exposure of the formulated phages to these conditions reduced their infectivity and in some cases, abolished their infectivity completely. The formulations developed for these phages are suitable to be used for field trials as the temperature is in the range of room temperature to 37 °C, with the soil pH 5-7. The use of protective formulations significantly increased bacteriophage treatment for disease control in the greenhouse. It was demonstrated that skim milk, rice flour and corn flour as potential carriers to protect the phage against the harsh environment. Moreover, the formulated phages displayed lower disease severity than the untreated and unformulated phages groups. Finally, the limitation of the study is that those two phages in treating which can infect all bacterial hosts of the same species should be investigated. In addition, the new potential carriers should be tested in future studies.

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