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Abstract: Fire blight, caused by *Erwinia amylovora*, is a devastating bacterial disease that threatens worldwide production of apples and pears. In Canada and the US, streptomycin and kasugamycin are commonly used for the control of this pathogen. There is an urgent need for development of biocontrol agents (BCAs) that can be easily incorporated into integrated pest management programs with high efficacy against streptomycin sensitive and resistant strains of *E. amylovora*. In addition, excessive use of antibiotics in agriculture has increased awareness of the possibility of antibiotic resistance gene transfer to human, animal and environmental microbes. To address this issue, a phage-carrier system (PCS) that combines *Pantoea agglomerans* as an antagonistic bacterial carrier of *Erwinia* phages as a double-pronged treatment for fire blight have been developed. The low viability of *P. agglomerans* cells following spray drying challenged the industrial scale production of this PCS. In the present research, a spray drying protocol was developed for *P. agglomerans* by modifying the growth medium and the bacterial cell formulation using D(+)-trehalose and maltodextrin. The developed protocol is amenable to industrial scale production of the BCA/PCS. The *P. agglomerans* viability was greater than 90% after spray drying, had a prolonged shelf life of 4 months at 4 °C, and the reconstituted cells showed an approximate 3 log reduction in *E. amylovora* counts with a green pear disc challenge assay.

Keywords: biological control agent; *Erwinia amylovora*; antibiotic resistance; streptomycin; apple; pear

1. Introduction

Fire blight, caused by *Erwinia amylovora*, is a devastating bacterial disease that threatens   apple and pear production worldwide [1]. The infection of the host by *E. amylovora* occurs during open bloom, when bacterial populations increase on the stigma. In the presence of water, the bacterial cells flow down into the hypanthium and reach the natural flower openings. Bacterial cells then invade the plant tissue, resulting in the infection of blossoms and shoots [2]. Antibiotics, such as streptomycin and kasugamycin, have been noted to have superior blossom blight control efficacy, with significant population reductions in *E. amylovora* on apple flower stigmas [3]. In Canada and the US, streptomycin and kasugamycin are the most commonly used pesticides against fire blight [4]. However, streptomycin resistant strains of *E. amylovora* have been identified in different countries around the world [5–8]. The importance of this is underscored by the fact that excessive use of antibiotics in agriculture has led to increasing awareness of the possibility of antibiotic resistance gene transfer to human and animal pathogenic microbes [9]. Currently, the European Union

regulatory agencies have restricted the use of streptomycin in agriculture, while Canada and the USA have only prohibited its use for organic farms [10].

Commercial biocontrol agents (BCAs) using antagonistic bacteria or lytic bacteriophages are available [9,11,12]. The BCA mode of action relies on the antagonistic interactions that result in competition for niches and nutrients and/or induction of plant defenses [13]. Commercially available bacterial-dependent BCAs against fire blight are available, such as BlightBan™ A506, Bloomtime Biological™ [14] and lytic phage-dependent BCAs such as, AgriPhage–Fireblight (OmniLytics, USA), have been authorized by the US Environmental Protection Agency (EPA) for fire blight treatment [15]. The phage-carrier system (PCS) is another form of BCA that combines antagonistic bacterial carriers with phages as a double-pronged treatment that was developed for fire blight control when the plants are blooming [16–19]. A study using PCS with *Pantoea agglomerans* and *Erwinia amylovora* phages showed a 56% reduced infection on flowers of potted apple trees, and this treatment compared well with the application of the antibiotic, streptomycin [20].

Spray drying and freeze-drying are the most widely used methods for producing BCA powders. The spray drying approach is preferable, as it is characterized by a high production rate and open production mode, as well as being easily scaled to industrial levels. Furthermore, the spray drying operation time and production costs are 30–50 fold lower when compared to the costly and time-consuming freeze-drying approach [21–23]. However, a significant drawback of spray drying is the loss of cell viability due to the use of high temperature and rapid dehydration during the spray drying process.

Previous spray dry trials with *P. agglomerans* showed low viability (3-29% viability) after spray drying due to the heat sensitivity of the cells [24]. The research presented herein describes an optimized formulation and protocol that leads to improved viability of spray dried *P. agglomerans*. Notably, the protocol demonstrated greater than 90% viability for both the *P. agglomerans* cells alone, as well as for this bacterium infected with *Erwinia* phages as a PCS. In follow-up experiments, a green fruit disc assay confirmed high efficacy of the spray dried *P. agglomerans* to control *E. amylovora* growth. Thus, the spray drying protocol developed herein provides a viable avenue to make industrial scale production of *P. agglomerans* BCA/PCS possible. Moreover, this protocol has broader implications for other antagonistic bacteria that are known to be sensitive to spray drying, given the easily adaptable formulation and protocol.

2. Materials and Methods

2.1. Bacterial Isolates

All bacterial strains used in this study are listed in Table 1. Cultures were stored at -80 °C in Microbank cryobeads (Pro-Bank Diagnostics, Richmond Hill, ON, Canada). To prepare the working culture stock, one Microbank cryobead was mixed with one drop of PBS buffer (10 mM, pH 7.2) and plated on 2.3% (w/v) Difco™ nutrient agar (NA) plates (BD, MD, USA). The plates were incubated for 16-18 h at 27 °C and then stored at 4 °C for 1 to 2 wks. Working cultures were obtained from the initial cultures by streaking single colonies onto NA and incubated at 27 °C for 16-18 h.

Table 1. Bacterial strains used for optimizing the spray drying method.

Strain	GenBank Accession #	Ref.
Pantoea agglomerans		
Pa39-7	JACSWZ000000000	[25]
Pa31-4	JACSXE000000000	[25]
Erwinia amylovora		
Ea6-4	JAAEVD000000000	[19]
EaD7	JAAEUT000000000	[19]

2.2. Bacteriophages

The *E. amylovora* bacteriophages used in this study are φEa21-4 (Myovirus) and φEa46-1-A1 (Podovirus) as listed in Table 2. To propagate each phage, a bacterial host (Table 2) suspension was prepared by suspending 5-6 colonies in 3 mL of 0.8% (w/v) nutrient broth (NB) (BD, MD, USA) to obtain an OD_{600} of ~ 0.6. Using a 250 mL baffled Erlenmeyer flask, 100 μL of the bacterial suspension was added to 75 mL of NB, which was incubated at 27 °C with 150 rpm shaking (New Brunswick Innova® 42, Eppendorf, Hamburg, Germany) for 3 -4 h. A 100 μL aliquot of phage stock (5.0×10^9 PFU/mL) was added, and the mixture was incubated for 16-18 h at 27 °C with 150 rpm shaking (New Brunswick Innova® 42, Eppendorf, Hamburg, Germany). Following incubation, 1 mL of chloroform was added to the culture and incubated with shaking for 5 min. The bacterial culture was centrifuged at 8500 x g at 4 °C for 15 min, the pellet discarded, and the supernatant filtered through a 0.22 μm Steriflip filter (Millipore, Billerica, MA, USA). The working phage stocks were stored at 4 °C in dark amber glass vials until needed and the titers were determined by using qPCR (Section 2.3) [19].

Table 2. *E. amylovora* phages used to generate a PCS.

Phage	Species	GenBank Accession #	Pathogen Host	Ref.
φEa21-4	*Kolesnikvirus* Ea214	NC_011811.1	Ea6-4	[18]
φEa46-1-A1	-	NA	EaD7	[18]

2.3. Quantitative PCR (qPCR)

Phage titers and *P. agglomerans* cell numbers were determined using qPCR using a protocol described by Gayder and coworkers (2019). Phage working solutions (or lysate) were treated with DNase I to remove non-encapsulated phage DNA prior to qPCR. DNase I treatments contained 10 μL of phage lysate, 1 μL of 2000 U/mL DNase I (NEB, MA, USA), 2 μL of 10X DNase I buffer (NEB, MA, USA) and 7 μL of water. The solution was incubated for 40 min at 37 °C and then 20 min at 80 °C. The qPCR 20 μl reactions consisted of 4 μL of 5X EVOlution Probe qPCR Mix (Montreal Biotech Inc., QC, Canada), 200 nM of each primer [19], 100 mM of the probe and 2 μL of the template. The reaction conditions began with 10 min at 95 °C, followed by 40 cycles of 10s at 95 °C and 45s at 54 °C. Dilutions of standard plasmid, pTotalStdA (10^{11}, 10^8, and 10^5 copies/mL), which contains amplicons for *E. amylovora* and *P. agglomerans*, and phages φEa21-4 and φEa46-1-A1 were included in every run to generate a standard curve. A linearly fit standard curve of Ct value vs copies/mL of the pTotalStdA was used to calculate the sample population quantities (genomes/mL). Populations of bacteria and phage were directly interpreted from this curve assuming that each cell or phage capsid contained a single genome. All qPCR quantification was performed with a Bio-Rad CFX96 qPCR System (Bio-Rad, CA, USA).

2.4. Bacterial Counting by Platting

Bacterial cell cultures or reconstituted suspensions were serially diluted in PBS and 100 μL of these dilutions was spread on NA and then incubated for 16-18 h at 27 °C shaking (New Brunswick Innova® 42, Eppendorf, Hamburg, Germany). Bacterial colonies were enumerated, and the final CFU/mL was calculated accordingly by taking into account the dilution and plating factors. Plating was carried out in triplicate.

2.5. Carrier Preparation

P. agglomerans was subcultured weekly on NA and, after 20 h at 27 °C, the plates were stored at 4 °C until needed that week. *P. agglomerans* cells were grown in NB as an initial rich-nutrient growth condition. For growth under stress conditions (*i.e.*, osmotic-adaptation), *P. agglomerans* was grown on medium that contains 5.0 g/L yeast extract, 10.0 g/L sucrose and 35.5 g/L NaCl (YSN media) or in NB with the same amount of NaCl to reach a water activity (a_w) in the medium of 0.98 a_w; where a_w is the

ratio between the vapour pressure of a sample compared to a control [26]. Cells were harvested by centrifugation (8500 x g for 10 min at 4 °C), resuspended in 10 mM PBS at pH 7.2 and enumerated using the serial dilution and spread plate technique on NA (Section 2.4). The colonies on the plates were counted after incubation for 16-18 h at 27 °C shaking (New Brunswick Innova® 42, Eppendorf, Hamburg, Germany).

2.6. Phage-Carrier Formulation

2.6.1. Polymer screening and Formulation Optimization

Different concentrations and combinations of polymers (all from Sigma-Aldrich, CA, USA) were used for formulations to explore with spray drying (Table 3). These polymers include D(+)-trehalose dihydrate, maltodextrin (dextrose equivalents 4.0-7.0 and 16.5-19.5), gum arabic (from acacia tree) and talc with carboxymethylcellulose sodium salt (CMC). *P. agglomerans* cell pellets were resuspended in a formulation mixture for 30 min to produce the formulated cells (f*Pa*).

The effect of formulation chemicals on the carrier infectivity by *Erwinia* phages was tested before selecting the suitable formulation for spray drying. Different concentrations of formulation chemicals were prepared in NB, as outlined in Table 3, and inoculated with *P. agglomerans* Pa39-7 (10^3 CFU/mL) together with the φEa21-4 phage (10^5 PFU/mL) in 96 well plates prior to incubation at 27 °C in a Synergy H1 microplate reader (BioTeK, Agilent, CA, USA) for 24 h. Readings of the plates at an OD_{600} were taken every 15 min and compared with the control sample (no phage added) in order to monitor growth/infectivity. After the initial trials, further optimization was carried out using different concentrations and combinations of the selected formulation chemicals as shown in Table 4.

Table 3. Polymers used for screening.

Chemicals	Final Concentration (%, w/v)
D(+)-Trehalose	0.05, 0.50, 5.00
Maltodextrin*	0.05, 0.50, 5.00
Maltodextrin*	0.05, 0.50, 5.00
Talc/CMC**	0.01, 0.10, 1.00

* Dextrose equivalents 4.0-7.0 and 16.5-19.5; ** CMC: Carboxymethyl cellulose.

Table 4. Concentration ranges used for optimization of the carrier formulation.

Chemicals	Final Concentration (%, w/v)
D(+)-Trehalose	13-15
Maltodextrin*	5-15
Talc	0-2
CMC**	0-1

* Dextrose equivalents 16.5-19.5; ** CMC: Carboxymethyl cellulose.

2.6.2. Spray drying of phage infected and non-infected carrier cells

Osmotically-stressed or non-stressed *P. agglomerans* cells (s*Pa* or ns*Pa*, respectively), were prepared by mixing cell pellets with a formulation solution (1g/100 mL) for 30 min before spray drying to form formulated stressed (sf*Pa*) or formulated non-stressed (nsf*Pa*). For phage infected cells (*i.e.*, the phage-carrier system*)*, *P. agglomerans* cell pellets were resuspended in PBS and mixed with the phage (φEa21-4 or φEa46-1-A1) at an MOI of 1.0 for 30 min. The cells were then harvested by centrifugation (8500 x g for 10 min at 4 °C) and resuspended in the formulation mixture (1 g/100 mL) for spray drying. The spray drying process was carried out using a Yamato ADL311S model (Yamato Scientific Co., Tokyo, Japan) with a feeding rate of 11-14 mL/min, inlet temperature of 160 °C and an outlet temperature of 60 °C. The spray dried powder was collected and stored in white plastic

polyethylene high density tubes [27] at 4 °C or room temperature (RT) with activated Silica gel packets (5 g each) (Millipore Sigma, CA, USA).

2.7. Storage Stability of the Spray Dried Preparations

Spray dried powder of phage infected, and non-infected carrier cells was collected and stored in polyethylene tubes (as mentioned above), then enclosed in Statshield® Moisture Barrier Al-bags (DESCO, CA, USA) and sealed with an Impulse Sealer, model AIE 300 (American international electric, CA, USA). Spray dried samples in sealed tubes were stored at RT or 4 °C for 16 wks (~ 4 months). At the time of sampling (biweekly), one tube was taken, and three technical replicates were prepared by reconstitution of the powder in sterile distilled water (0.9 g/ 3 mL). *P. agglomerans* cells were enumerated by plating on NA, and the logarithmic cell counts were plotted versus time. For phage enumerations in PCS powder, 100 μL of reconstituted powder was used to inoculate 10 mL of NB and incubated at 27 °C with shaking at 180 rpm shaking (New Brunswick Innova® 42, Eppendorf, Hamburg, Germany). The phages were enumerated by qPCR at time intervals of 0, 2, 4 and 18 h (T_0, T_2, T_4 and $T_{O/N}$). To test the viability of the phage in the reconstituted PCS powder, 100 μL volume of a 1/10 dilution of the reconstituted powder was platted on NA plates and incubated for 16-18 h at 27 °C. The plaques formed by active phages were counted and PFU/mL was assessed after taking into account the dilution and plating factors.

2.8. Efficacy Testing of Phage-Carrier with a Green Pear Disc Assay

Green pears were purchased from a local grocery store, washed, rinsed, surface sterilized with 70% (v/v) ethanol and rinsed twice with sterile distilled water. Under sterile conditions, a copper borer was used to cut into the fruit and produce pear plugs from which discs were cut measuring 1.5 mm in diameter and 1 mm in depth. The discs were placed into 2 mL sterile Eppendorf tubes using sterile forceps. Treatments consisted of formulated *P. agglomerans* (Pa39-7, formulated *P. agglomerans* plus phages (Pa39-7 + phage φEa21-4), a positive control (1:1 ratio of *E. amylovora* strain Ea6-4 and D7) and non-infected formulated *P. agglomerans* (negative control). Two biological replicates, containing eight technical replicates were set up for each treatment. To each pear plug, 10μl of treatment was added and sample tubes were collected at 0, 24 and 48 h. A 10 μL volume of pathogen was added at 10^6 CFU/mL at 24 h and allowed to incubate for an additional 24 h. Following this time period, 500 μL of 0.01 M phosphate buffer pH 7.0 was added to the tubes containing the disc and sonicated for 3 min at high power for 3 min. A 200 μL volume of the sonicated supernatant was removed into 2 mL sterile Eppendorf tubes and the cells were heat killed at 80 °C for 10 min. The heat-treated supernatant was frozen at - 20 °C prior to evaluation by qPCR. A Student's t-test was performed comparing the means between the 24 and 48 h experimental data. Statistical analyses were performed using the JMP programme (JMP Statistical Discovery LLC, Cary, NC, USA). A three-way random analysis of variance (ANOVA) was performed on natural log (ln) + 1 transformed data comparing the effects of two formulated products at suppressing the population of E. amylovora in vitro on green pear discs over time.

2.9. Powder Physical Properties

2.9.1. Water activity (a_w)

Carrier and PCS powder water activities were measured by using an Aqualab 4TE water activity meter according to the manufacturer's instructions. Three technical replicates of powders were prepared and tested for the shelf-life experiment.

2.9.2. Transmission Electron Microscopy (TEM)

Copper–rhodium (150-mesh) grids were covered with a thin layer of amorphous carbon made hydrophilic by a 45 s vacuum glow-discharge. PCS powder was rehydrated in filtered MilliQ water and then 4–6 μL were placed on individual grids and left for 2 min to adsorb. Excess sample was

gently removed by filter paper. The grid was then washed three times with water. Sample on the grids were stained with 2% (w/v) uranyl acetate. A Tecnai G2 transmission electron microscope (FEI Company, Hillsboro, OR, USA) at the University of Guelph Electron Microscopy Unit (Guelph, ON, Canada) was used for visualization, operating at 200 kV under variable magnification (x 50,000). Images were acquired using the Gatan Ultascan 4K CCD (Pleasanton, CA, United States) and Gatan Digital Micrograph software imaging system.

3. Results

3.1. Polymer Selection for the Phage-Carrier Formulation

To exclude the possibility of interference of the formulation chemicals on phage infectivity of the carrier cells after reconstition, the killing of *P. agglomerans* cells by φEa21-4 phage was assessed by turbidity measurements of cultures in the presence of each formulation chemical being tested. Phage infectivity and growth of carrier cells was not significantly affected by the presence of D(+)-trehalose (Figure 1A) or maltodextrin (Figure 1B). Interestingly, the presence of D(+)-trehalose or maltodextrin actually augmented the ability of the phage to infect the host cells. Similarly, presence of Talc/CMC did not affect the growth of the carrier cells or its infectivity φEa21-4 phage (data not shown). Based on the formulation screening results, 20% (w/v) D(+)-trehalose with 2% (w/v) talc and 0.1% (w/v) CMC, were selected as formula I for cell preparations before spray drying.
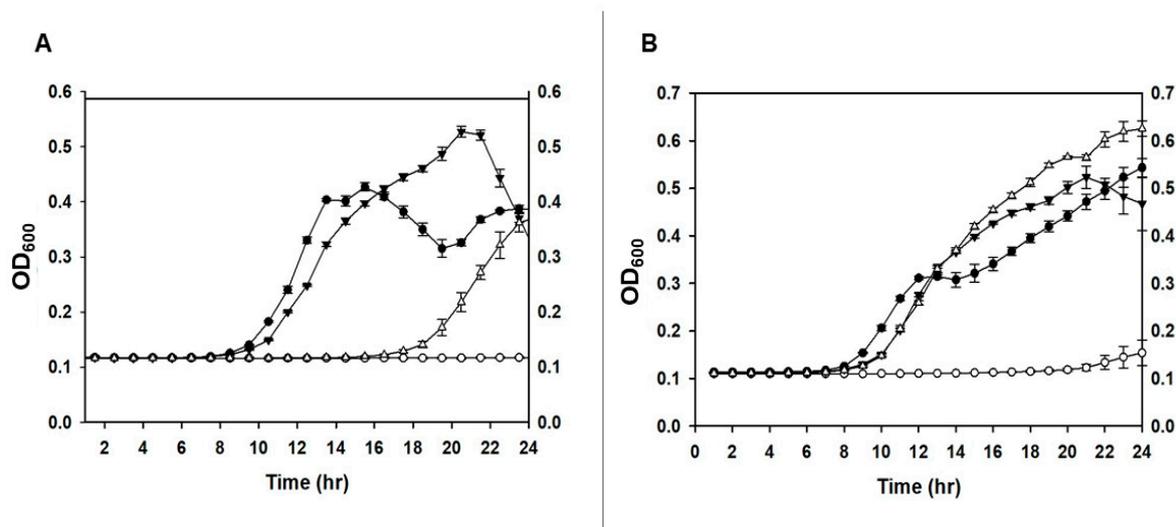

Figure 1. Determination of φEa21-4 phage infection of *P. agglomerans* cells in the presence of different formulation chemicals. A) Survival *of P. agglomerans* Pa39-7 strain after phage φEa21-4 infection in the presence (O) and absence (Δ) of D(+)-trehalose. Uninfected *P. agglomerans* Pa39-7 growth in the presence (•) and absence (▾) of D(+)-trehalose was also measured as controls. B) Survival of *P. agglomerans* Pa39-7 strain after phage φEa21-4 infection in the presence (O) and absence (Δ) of maltodextrin. In the absence of phage, *P. agglomerans* Pa39-7 growth in the presence (•) or absence (▾) of maltodextrin was similar. Assays were carried out in triplicate and the data averages and standard deviation were plotted.

Despite the promising results, there were downstream application troubles using formula I. Following reconstition of the spray dried powders for the biocontrol assay, it was observed that the atomizer nozzle became clogged by the talc particles. Hence, further optimization was carried out using different mixtures of polymers, as shown in Table 4, to solve the clogging of the nozzle. Accordingly, formula II was a modified version containing 15% (w/v) of D(+)-trehalose and 15% (w/v) maltodextrin 16.5-19.5. This represented the final formulation that was used in all other experiments.

3.2. Optimization of Spray Drying and Reconstitution Protocols

Several trials were carried out to determine the optimal spray drying temperatures and reconstitution times of the spray dried powders. Experiments were conducted at different inlet (150-160 °C) and outlet (50-60 °C) temperatures to assess the parameters that minimize the loss in viability of the carrier cells during the spray drying process. According to this optimization, the best combination of inlet and outlet temperatures were determined to be 160 and 60 °C, respectively. After spray drying, the powder produced was dissolved in sterile distilled water to 0.3 g/mL to retain the same weight/volume ratio used before the spray drying process. Optimum reconstitution time was assessed by the powder solubility and cell plating on NA across a time course.

3.3. Confirmation of Cell Viability and Phage Infectivity After Spray Drying

Once the formulation and spray drying process was determined, the viability of formulated *P. agglomerans* carrier cells that grew in NB (nsf*Pa*) were compared to osmotically-adapted cells grown in NB plus NaCl medium. The nsf*Pa* formulation displayed a 30% reduction in cell viability after spray drying (Figure 2). However, osmotically-adapted cells showed ≥ 95% of cell viability for carrier cells (sf*Pa*) and about 90% for the PCS infected-carrier (sf*Pa*-P), as shown in Figure 2. The presented results are for *P. agglomerans* strain P39-7 and φEa21-4 phage, however, strain Pa31-4 showed the same cell viability as Pa39-7 after spray drying. Also, the cell viability after spray drying of Pa39-7 strain infected with φEa46-1-A1 was similar to that infected with φEa21-4 phage.
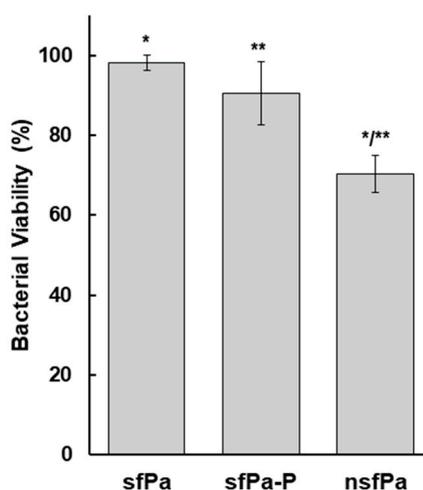

Figure 2. Viability of *P. agglomerans* after spray drying. The viability of spray dried *P. agglomerans* was measured as a percentage by comparing after and before spray drying cell counts. All tested *P. agglomerans* cells were formulated by resuspension of cell pellets in the formula II. Both versions of the osmotically-adapted cells that grew in NB plus NaCl medium, either phage-uninfected (sf*Pa*) or φEa21-4 phage-infected (sf*Pa*-P), showed greater than 90% viability after spray drying compared to osmotically non-adapted cells (nsf*Pa*), which showed about 70% cell viability. Assays were carried out in triplicate and the data averages and standard deviation were plotted. Using a t-test, the significance ($P < 0.05$) between sf*Pa* and nsf*Pa* (*), as well as between sf*Pa*-P and nsf*Pa* (**) was noted.

A 1/10 dilution of reconstituted PCS powder (*ie.*, from the sf*Pa*-P cells) was plated onto NA to mimic the double-layer plaque overlay assay to test the phage infectivity in the phage-carrier powder. After a 24 h incubation at 27 °C, plaques were observed (data not shown), indicating the presence of active phage in the powder of spray dried infected carrier cells. Furthermore, the phage titer increased by more than 4.5 log (PFU/mL) after 24 h when the reconstituted PCS powder was used to inoculate 10 mL of NB, as shown in Figure 3.
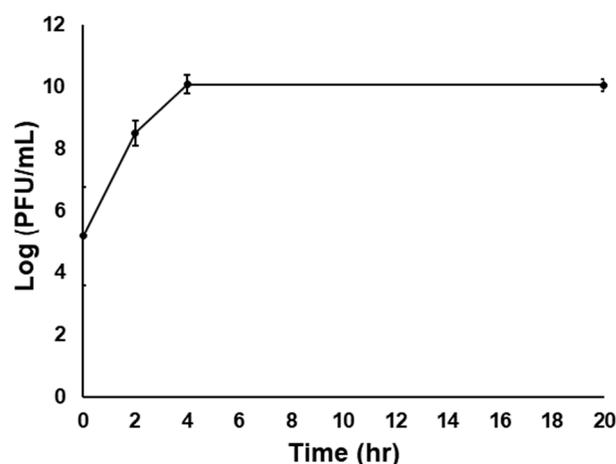
Figure 3. Infectivity of φEa21-4 phage following reconstitution of the spray dried powder. A volume of 100 μL of the reconstituted PCS powder was used to inoculate 10 mL of NB and incubated at 27 °C with shaking at 18 rpm. The graph displays the phage titer (Log PFU/mL) increased with incubation time and that the phages are viable. Assays were carried out in triplicate and the data averages and standard deviation were plotted.

The water activity of the powder (a$_W$) was found to be 0.18±0.002. On the other hand, TEM images were taken to compliment the *P. agglomerans* viability and phage plaque assays with reconstituted PCS powder. The images show assembled phage particles (red arrows) inside and outside of the carrier cells (Figure 4a and 4b, respectively). Overall, these results further confirm that osmotic adaptation in combination with formula II act to protect the phage and carrier cells during the spray drying process so that they are present and viable after storage and reconstitution.
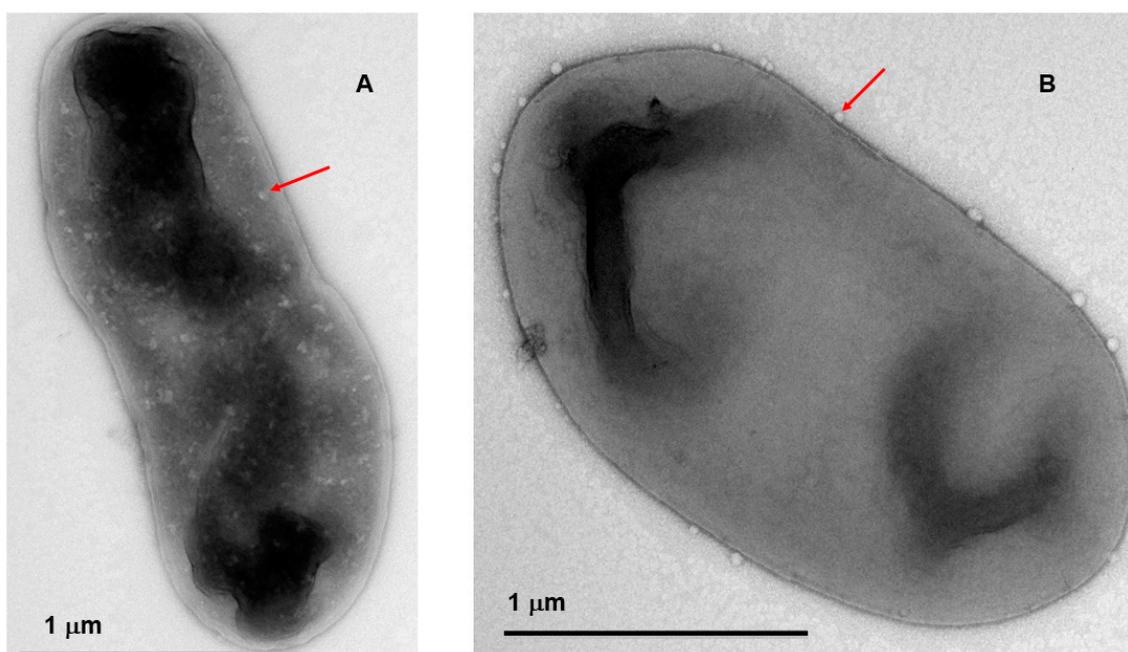

Figure 4. Electron microscopy of φEa21-4 assembled phage particles after reconstitution of the spray dried powder. Transmission electron microscopy images of the PCS reconstituted powder samples showed phage particles assembled inside (A), or phage attached to the cell (B).

3.4. Shelf-life of the Carrier and Phage-Carrier Spray Dried Powders

Cell count and phage infectivity assays were conducted every 2 wks for 4 months to assess the carrier and phage viability in the powder following storage. Viability *of P. agglomerans* in the carrier powder was reduced by more than 2 log (CFU/g) after 16 wks of storage at RT. However, only a 1-1.5 log reduction in bacterial counts was observed in powder stored at 4 °C (Figure 5A). For the PCS powder, *P. agglomerans* viability was reduced by 4 log CFU/g after 16 wks at RT and maintained an approximate 2.5 log reduction when stored at 4 °C (Figure 5B). Inoculation of reconstituted PCS powders in NB to test phage infectivity gave a similar pattern that mentioned before in Figure 3.
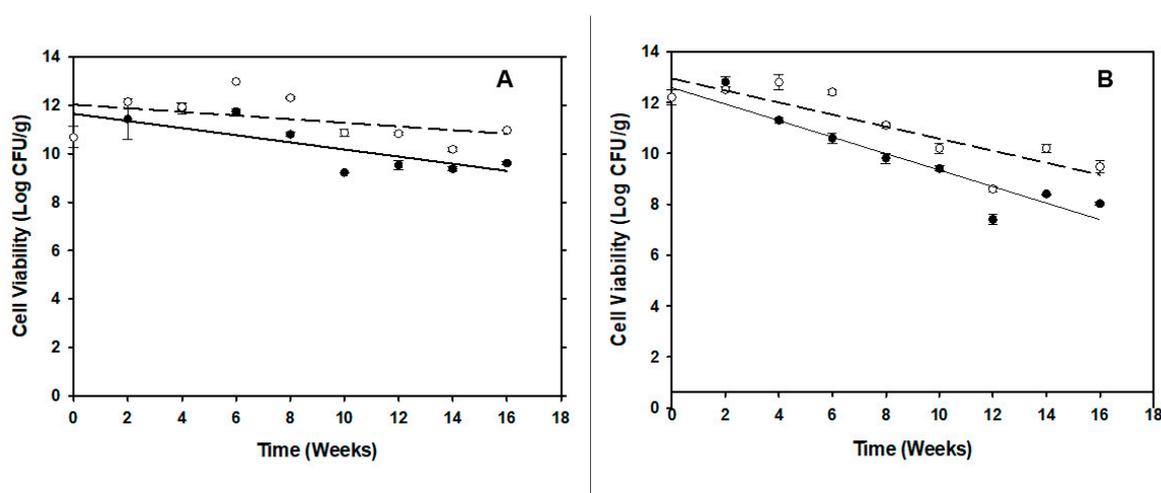

Figure 5. Changes in cell viability of *P. agglomerans* Pa39-7 cells in BCA or PCS powders after storage. A) Changes in the cell viability of carrier cells in BCA powder stored at 4 °C (O) or at RT (●). B) Changes in the cell viability of carrier cells in PCS powder stored at 4 °C (O) or at RT (●). Assays were carried out in triplicate and the data averages and standard deviation were plotted.

3.5. Phage-Carrier Efficacy Assessment with the Pear Disc Assay

A pear disc was used as an in vitro assay to assess the protection that the BCA and PCS powder formulations can afford to plants when challenged with E. amylovora. In these trials, the BCA (only P. agglomerans) and PCS (P. agglomerans and phage ϕEa21-4) were not significantly different from each other in population growth during the 24 h and 48 h sampling on the pear discs (Figure 6; c and a respectively). Using this assay, E. amylovora was also noted to grow on the pear discs and increased by 2 log between the 24 h to 48 h time points (Figure 6; f and b respectively). When the myovirus ϕEa21-4 population was monitored on the pear discs, a slight reduction in titer at 48 h after being exposed to E. amylovora for 24 h was noted (Figure 6; d to e comparison). When the *E. amylovora* population was followed by qPCR, both the BCA (Pa39-7) and PCS (Pa39-7 + ϕEa21-4) led to substantial significant reductions in E. amylovora numbers at the 48 h timepoint (Figure 6; g and h compared to b). Interestingly, BCA was noted to have a slightly more significant effect in suppressing the E. amylovora pathogen on the green pear discs than PCS under these assay conditions (Figure 6; g and h respectively).
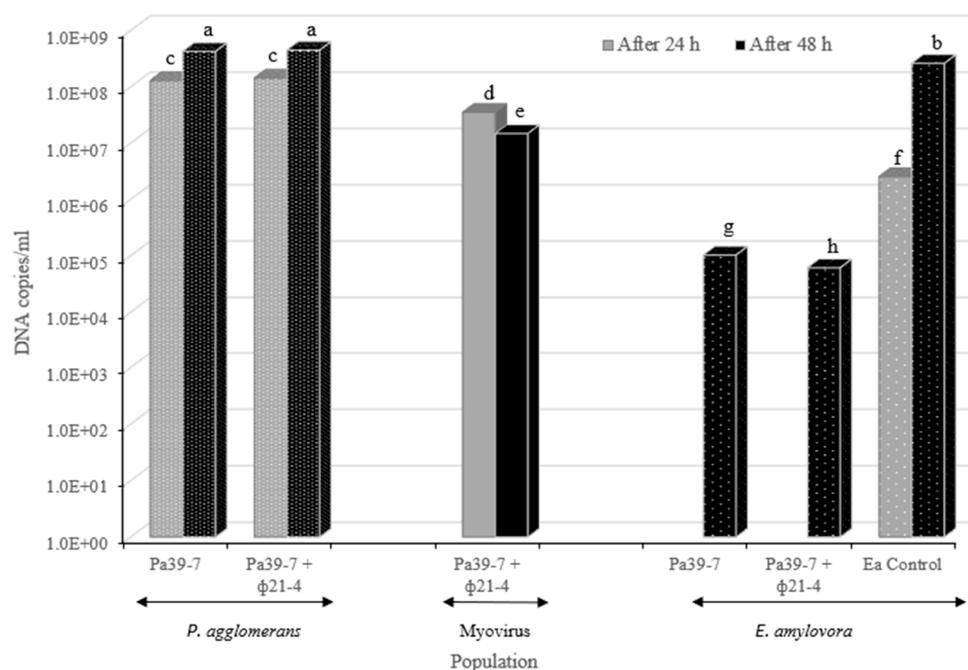
Figure 6. Populations of *P. agglomerans*, φEa21-4 (Myovirus), and *E. amylovora* on green pear discs measured at 24 and 48 h. On the x-axis, application to the pear discs of the BCA (*i.e.*, containing only *P. agglomerans* strain Pa39-7) is denoted by Pa39-7, the PCS (*i.e.,* containing φEa21-4 phage-infected *P. agglomerans*) is denoted as Pa39-7+ φEa21-4 and only *E. amylovora* as Ea Control. The organism determined by qPCR in each of the assays is indicated below the x-axis labels with black arrows. Grey bars represent populations on the pear discs at 24 h. For the c and d samples, this is the bacterial counts before inoculation with *E. amylovora*, while in the Ea Control (f) this represents the *E. amylovora* numbers at 24 h. All bars at 48 h (black) represent the growth of each organism in each formulation after 24 h of additional growth in the presence of *E. amylovora*. Analyses showed the interaction between all variable combinations in the experiment were highly significant (p < 0.0001). Replicate and random effects in the analysis were not significant (p > 0.05) indicating there was no variability between replicates of individual groups. .

4. Discussion

In fire blight management, BCAs and phages (registered in the USA) are commercially available as alternatives to antibiotic utilization [9,14,15]. Subsequently, PCS was developed as an approach that combines the biological activity of antagonistic bacteria, *P. agglomerans*, and *Erwinia* phages that specifically target and kill the fire blight pathogen [16,17,28]. An optimized and economical production protocol is required for industrial scale production of the PCS and spray drying or freeze-drying are the most widely used methods for production [21]. However, freeze-drying is costly, time consuming and operates as a closed system, which means that it cannot be used in a continuous largescale production mode. In contrast, spray drying has a higher powder production rate, continuous production mode, is easily scalable to industrial levels and is 30-50 fold more cost effective compared to freeze-drying [21–23]. The major drawback of spray drying is the loss of cell/phage viability due to exposure to high temperatures during preparation. For example, *P. agglomerans* carrier cells are thermally sensitive and have exhibited a significant reduction in cell viability (only 3 - 30% cell viability following spray drying) [24,29,30]. Bacterial growth under osmotically-adapted conditions is known to increase tolerance to desiccation. More specifically, osmotically-adapted *P. agglomerans* can accumulate glycine-betaine, ectoine and D(+)-trehalose [26,30,31]. Under this premise, osmotically-adapted (*i.e.*, grown in a standard medium with NaCl to a water activity of 0.97-0.98) and spray-dried *Pantoea* were able to consistently display cell viabilities around 30% [24]. However, these low viability numbers remain a hindrance to industrial scale production.

This study established an improved *P. agglomerans* viability protocol by optimizing the formulation mixtures, spray drying temperatures, nozzle clogging and reconstitution times. The culmination of this work indicates that *P. agglomerans* (either under non-stressed or osmotically non-adapted conditions) treated with formula II have approximately 70% survival after spray drying. Further optimization in the introduced protocol was carried out by growing *P. agglomerans* on NB with NaCl at an a_w of 0.98 for 18-20 h. This osmotic-adaptation further improved cell viability to more than 95% for *P. agglomerans* alone (BCA) or more than 90% for PCS, which far exceeds the previously published results of ~30% for osmotically adapted [30] and 3% for non-adapted [24]. Thus, this enhanced protocol makes it a very promising approach for industrial scale production of *P. agglomerans* as BCA or PCS applications to control fire-blight, but also more widely as an avenue to explore for other bacteria recalcitrant to these processes.

For the PCS, the modified protocol was also assessed for survival and infectivity of the phage in the carrier bacteria. In the presence of D(+)-trehalose and maltodextrin the survival of *P. agglomerans* with the phage was markedly lower; thereby suggesting that not only had phage φEa21-4 survived the spray drying process, but that the presence of formula II components enhanced its infectivity (Figure 1A&B). The presence of formulation compounds has been suggested in the literature to increase the viscosity and hence phage adsorption and/or can lead to changes in the bacterial metabolism [32,33]. While plausible as a reason for the observed increase, further research would be needed to confirm these possibilities in our studies.

Maintaining the viability of the carrier and the ability of these cells to be infected with *E. amylovora* phages are important factors for a suitable PCS. In this work, formula II was noted to protect both phage-infected and non-infected cells, as shown by a combination of plaque assays, NB inoculation with reconstituted powder, as well as electron microscopy imaging results (Figure 3, Figure 4). In addition, the powder storage protocol used in this study utilized low O_2-permeability tubes, silica gel packets to control humidity, and enclosure of the bottle in Statshield® Moisture Barrier Al-bags, along with different storage temperatures. Combing these storage conditions led to high cell viability of both carrier and PCS powders for almost 4 months (*i.e.*, only 1-2 log reductions in viability over this period). By this process, high humidity content in the powder, which can cause the crystallization of the amorphous trehalose matrix and decrease the cell viability [34,35], appears to have been mitigated. Our low temperature results are also consistent with other studies that demonstrated that powder storage temperature has previously been noted to be an important factor that can affect the viability of the cells during storage due to membrane lipid oxidation [36,37]. Thus, by combining all of these factors we have been able to generate a PCS that has proven longevity that may be amenable to production and delivery for field applications.

As an initial *in vitro* test of the efficacy of the BCA and PCS powders in mitigating *E. amylovora* infections, reconstituted powders were used on pear slices as a model system of infection. Both the BCA and PCS preparations were able to decrease *E. amylovora* growth by 3 log reduction compared to untreated samples after 24 h of infection time with *E. amylovora*. Interestingly, the populations of carrier and phage (in the case of PCS) were not significantly changed over the entire 48 h experiment. These results are in contrast to our separate experiment where reconstituted PCS in NB led to a doubling of the phage counts after 24 h with shaking at 27 °C (Figure 3). It is plausible that there are alterations or delays in the infection process for the phage in the pear disc assay given that these environmental conditions are likely to be different than the rich NB media. Indeed, published data has noted that there are several factors that can contribute to discrepancies in phage counts between assays. For example, the bacterial carrier metabolic state can play a vital role in phage adsorption/infectivity and consequently phage propagation. For instance, the burst size of T4 phage is dependent on the cell state of *E. coli* strain B23 [40]. T4 burst size can vary between 1-40 progeny/cell depending on whether they are in the mid-log or stationary phase of growth, where the latter may produce only one phage per cell since the cells have entered a hibernation of scavenger response mode[41]. The cell's metabolic state can also influence the sigma factors present within cells, which can differentially orchestrate the activity of the DNA-dependent RNA polymerase (RNAP) holoenzyme (Eσ) during the transcription process [42]. For example, *E. coli* contains six σ factors,

including σ^{70} that is present during exponential growth and σ^S that is found during the stationary phase [42,45] and activated by various stress signals, including hyperosmolarity [46]. In the present spray drying protocol, *P. agglomerans* was exposed to salt stress during osmotic adaptation and stationary phase growth, which combined with further phyllosphere pressures/responses led to slower/lower release of phages and concomitantly higher numbers of *P. agglomerans*. While the PCS and BCA both showed antagonistic behaviour towards *E. amylovora*, the pear disc assay highlights the importance of assaying beyond the typical *in vitro* rich culture scenarios and provides a future avenue to explore observations over longer time periods, MOI concentrations, reconstitution variables and more.

5. Conclusions

An improved viability protocol for spray dried *P. agglomerans* as a BCA and PCS was established. This protocol can be used for industrial scale production of *P. agglomerans* BCA or PCS. Given the efficacy of this protocol, it is likely to also have broader applicability to other thermal sensitive BCAs in development as an example of iterative optimization that can have drastically different yields and improved cell viability from 3-30% to over 90%.

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