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Bacteriophage As a Promising Biocontrol Agent for *Pectobacterium Carotovorum*, Inciting Potato Soft Rot Disease

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Potato, *Solanum tuberosum*, soft rot, *Pectobacterium carotovroun* subsp. *carotovorum*, phage therapy, bacteriophages.

ABSTRACT

Pectobacterium carotovorum subsp. *carotovorum* (*Pcc*) is the main causative of soft rot disease in a vast variety of vegetables and crops worldwide. Currently, phage therapy used to be a safe biological opportunity for manipulating plant bacterial infections. Hence, this study was designed to isolate and identify *Pcc* isolates and their specific phages. Twenty *Pcc* isolates were isolated from diseased potato roots and soil cultivated with various crops. *Pcc* isolates were identified based on morphological and biochemical characteristics. Pathogenicity studies revealed that healthy potato tubers were responsive to soft rotted *Pectobacterium*. Twenty-three specific bacteriophages for isolated *Pcc* were isolated from infected potato tubers, soil and sewage and drainage water samples and then, identified via spot and plaque assay techniques. The phages confirmed various morphological plaques ranging from 1.0 to 5.0 mm in diameter, then purified for establishing the morphological properties using TEM and genetic variability using restriction enzyme pattern. The obtained phages have the ability to prevent biofilm formation of *Pcc* isolates with percentages of 98.5, 81.0, 81.6 and 97.0 for *Pcc*1, *Pcc*2, *Pcc*3 and *Pcc*4, respectively. Applying of phages cocktail on potato tubers caused a reduction in soft rot indications and disease severity by about 7.0, 15.0, 17.5 and 15.0%. Nevertheless, the short longevity of phages on host plants is considered one of the obstacles to their application. So, some phages formulations were prepared to increase their longevity, among them the formula containing phages, corn flour (0.5%) and sucrose (5%) (v/w) was the most efficient one for improving phage longevity at room temperature and decreased the disease severity to 5.0, 10.0, 15.0 and 13.0% for *Pcc*1, *Pcc*2, *Pcc*3 and *Pcc*4, respectively compared to *Pcc* isolates on potato tubers.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is considered an economically common edible plant with high importance in moderate climates, more rarely in warm ones. On a global scale, the potato is considered the 4th most cultivated food crop after wheat, rice, and maize. In Egypt, the potato has a distinctive place among all vegetable crops and is put to use in

human consumption and animal feed (FAO, 2019). Potato tubers are attacked by a different bacterial pathogen, the most important of which is *Pectobacterium carotovorum*, which causes soft rot disease, and also is the cause of soft rot disease in many vegetables and crops. (Russell, 2008 and Surman, 2011). It occurs on plant surfaces and soil and invades the host by wound sites or natural openings during transportation and storage. After successfully invading the plant, it reaches into vascular tissues and intercellular spaces, then secretes plant cell wall-destroying enzymes (PCWDEs) within the disease-promoting environment. During pathogen infection, extracellular maceration enzymes, for instance, polygalacturonase (Pg), pectate lyase (Pel), cellulase (Cel), xylanase and protease (Prt) enzymes can degrade plant structures consisting of pectin, cellulose and hemicelluloses fibers ultimately causing plant cell necrosis and tissue maceration (Abbott & Boraston, 2008 and Czajkowski *et al*., 2011). Several strategies were introduced to help reduce the impact of the disease. Chemical control methods along the lines of pesticides, antibiotics and copper are used. The continuous using of such a method brings about a toxic effect on the environment (Clark *et al*., 2006). Recently, biological methods are applied for the control of various plant diseases due to their safety for health and the environment. Bacteriophages, which are bacteria's natural predators, have been discovered to be effective in modern biotechnology. Using of bacteriophages as bio-therapeutic agents are well known over the world. Phages have also been approved in order to use as bio-therapeutics in food and plant diseases (Shirley, 2016). Phages are small viruses that existed in practically overall environments and show high specificity for bacteria without having any direct adverse reactions on treated animals or plants. Aside from that, the bacteriophages are believed to be bactericides rather than bacteriostatic, and in addition, they act against antibiotic or heavy-metal-resistant bacteria with high specificity biofilm disruption (Sadik *et al*., 2014 and Shirley, 2016). However, (Tewfike *et al.,* 2016 and Anna *et al*.,2020) revealed that viruses are extremely fragile and cannot reside long on plant surfaces because of harmful environmental factors such as rain, sunlight UV and moisture which quickly eliminate them. Therefore, the necessity arose in order to develop some formula (phages, corn flour and sucrose) to change the application strategy to increase the efficacy of phage treatments and to some more convenient application schedule (Balogh and Jones, 2003).

The aim of this research is an attempt to reduce or slow down the incidence of potato soft rot disease at room temperature by using phages, in addition to finding new protective formulations in order to increase the longevity of phages.

MATERIALS AND METHODS

Isolation and Purification of a Causal Bacterium:

Isolation of the targeted causal bacterium was done from tubers of naturally infected potato (*Solanum tuberosum*) plants cv. Spunta is cultivated on the farm of the Faculty of Agriculture, Qalubiah governorate. The collected potato tubers showed distinctive soft rot symptoms. The pathogenic bacteria were isolated by spread plate method on nutrient agar medium and then purified by streak plate method on nutrient agar medium (Difco). The purified bacterial isolates were inoculated on crystal violet polypectate as a selective medium and then cultured on King B and Raffinose media for identification. Purified isolates grown on the above-mentioned media were preserved on slants of the nutrient agar medium at 4°C for subsequent identification. Isolates were maintained at 4°C in a nutrient broth-glycerol mixture (nutrient broth containing 20% glycerol) for preservation. Identification of bacterial isolates was carried out according to Bergey's, (2005) as well as using PI 20E strips for Enterobacteriaceae.

Isolation and Purification of Bacteriophages:

The effective phages against isolated *Pectobacterium* (*Pcc*) were detected from collected infected potato tubers, soil and drainage water samples according to the method of Othman (1997). Ten ml from each sample were added to Erlenmeyer flasks (250 ml) containing 100 ml of nutrient broth medium and inoculated with 10 ml of each isolate of *Pcc* were mixed thoroughly. After that, the cultures were centrifuged at 6000 rpm for 15 min, then the supernatants were collected in clean flasks. Chloroform was added at a rate of 1:10 followed with shake vigorously for 5 min., the crude lysate belonging to the phages was obtained and assayed qualitatively and quantitatively in accordance with Borrego *et al*., (1987).

Determination of Dilution Endpoint (DEP) For Isolated Bacteriophages:

The dilution end point of *Pcc* phage isolates was determined by plaque assay technique according to Maniastis, (1982). Plates were incubated at 30˚C for 24 hrs.

Storage Stability:

The infectivity of *Pcc* phage was examined by spotting phage lysate daily after incubation at different temperatures, 4, 37 and 40˚C for 30 days using the spot test technique (**Feisal *et al*., 2013).** The stability of formulated phages (Phages+0.5% corn flour+5% sucrose) was assayed daily by spot test at room temperature for five weeks.

Determination of Host Range Pattern of The Isolated Bacteriophages:

The host range of the isolated *Pcc* phages was determined using the spot test as described by Sambrook *et al*., (1989). *Pectobacterium carotovrum* isolates, *E. coli*, *Pseudomonas* sp., *Xanthomonas* sp., *Bacillus* sp. and *Serratia liquefaciens* were used during this experiment. The plates were incubated for 24 hrs at 30˚C for detection of a clear zone of bacterial lawn completely lysis.

Morphological Properties of *Pcc* Phages (Transmission Electron Microscopy):

One drop of isolated *Pcc* phages suspension was placed on a 200-mesh carbon-coated copper grid and allowed to absorb for approximately 20 min. The grids were negatively stained with 2% uranyl acetate (pH 4.5) for 90 sec. and left for drying and then examined using a JOEL-1010 electron microscope operated at 60 KV as mentioned by Luftig, (1967). The morphological properties of the isolated *Pcc* phages were identified and carried out using a transmission electron microscope at the Regional Centre of Mycology and Biotechnology, Al-Azhar Univ., Cairo.

The Anti-Virulence Activity of *Pcc* Phages:

- **Quantitative Biofilm Formation Assay:**

The tissue culture plate method was used as a quantitative test described by Christensen *et al*., (1985). The wells of 96-well flat-bottom polystyrene micro-plates (Sigma-Aldrich, Costar, USA) were filled with 200 µl of diluted cells per well, of the diluted cultures to a cell density equivalent to an OD_{600} of 0.2–0.3 (~10^8 CFU/ml). Negative control wells were inoculated with 200 µL sterile broth medium. The plates were incubated at 30˚C for 24 hrs. After incubation excess, free-floating bacteria in each well were eliminated carefully. Wells were rinsed with 0.2 ml of saline phosphate buffer (pH 7.2) four times. Biofilm produced by bacteria adhered to the wells was fixed with 2% sodium acetate and stained with 0.1% crystal violet for 20 min. The excess stain was removed by means of deionizer water and plates were kept for drying. To quantify the amount of biofilm, the optical density sample was obtained using ELISA microtiter-plate reader (Sun Rise–TECAN, Inc. ®, USA) at a wavelength (570 nm). The experiment was performed in triplicate and repeated three times. The interpretation of biofilm production was done according to the criteria of Lee *et al*., (2013).

- **Biofilm inhibition assay:**

The *Pcc* phages' potential to stop biofilm creation of *Pcc* isolates was tested at sub-dilution endpoint concentrations. Tested phage of two-fold serial dilutions was prepared in the good microtiter plate containing nutrient broth with 2% glucose. Bacterial suspensions (50 µL; 5×10^8 CFU/mL, final concentration) were then transferred into the plate. Following incubation at 30°C for 24 hrs, the effect of the tested phage on the bacterial growth was evaluated using the microplate reader at an optical density of (570 nm). The bacterial biofilm formation in the presence of the tested phage was subsequently determined and compared with the positive control (Lin *et al*., 2011).

Phage-Therapy of Bacterial Soft Rot Disease on Potato Tubers:

- **Virulence Test:**

Healthy potato tubers cv. Spunta obtained from Vegetable Crops Res. Dept., Agric. Res. Cent. (ARC), Giza, Egypt, were used during this experiment as well as four Pcc isolates were tested. The tubers were divided into four groups; each group contained ten tubers incubated for 7 to 30 days at room temperature after being treated as follows:

- Group (1): inoculated with bacterial suspension (10^8 CFU/ml) using a hand-held plastic sprayer until completely wet and considered as a positive control.
- Group (2): inoculated with phage cocktail until completely wet and considered as a negative control.
- Group (3): inoculated with bacterial suspension and sprayed with unformulated phage cocktail suspension.
- Group (4): inoculated with bacterial suspension and suspension of formulated phage cocktail.

Disease severity was carried out two times during the incubation period according to **Weller (2007)** and calculated the soft rot areas.

- **Determination of the Phage Titer:**

The weight of tubers before treatment and after the rotten tissue was scraped off were calculated according to Balogh and Jones (2003). After incubation periods for three tubers samples of each treatment, the soft rot was placed in the plastic bags and kept in the ice box and weighted and poured 100 ml of deionized water into the bag. Plaque assay was done to calculate the phage titer as PFU per gram of potato tubers by the following equation.

$$\textbf{Phage titer} = \frac{\text{Plaque number} \times 1.000}{\text{weight of sample bag} - \text{weight of empty bag}}$$

RESULTS

Identification of Bacterial Isolates:

Twenty isolates that recovered on nutrient agar medium and were found to have circular, creamy, convex, smooth and entire margins were identified as *Pectobacterium carotovorum* (*Pcc*). Furthermore, all isolates were able to grow and form pits on CVP media as well as they were creamy in color with no fluorescent pigments on King B medium and small dark red colonies on Raffinose-based medium. Moreover, the bacterial cells were observed as rod-shaped, Gram-negative, non-spore-forming, motile and didn't able to produce pigments in the medium.

According to the biochemical characteristics, results indicated that 20 isolates grown at 37°C, 5% and 7% NaCl, and gave positive reactions for KOH, pectinase, catalase, methyl red, Voges Proskauer (acetone production), casein and gelatin hydrolysis, H_2S production, Citrate (Simmon) and acid production from glucose, sucrose, fructose, lactose, cellobiose, raffinose tests. On the contrary, all isolates gave a negative reaction for oxidase,

indole, urease, starch hydrolysis, and acid production from maltose tests. API test strips (analytical profile index) were used as a biochemical system for the identification of Enterobacteriaceae and confirmed that the isolates were *Pcc*.

Identification of Specific Phage for *Pcc*:

Pcc phages detected in the crude suspension of potato rotted tubers, rhizosphere soil, and sewage water and gave positive results by the spot test (Fig. 1.a) were assayed quantitatively by the plaque assay technique (Fig.1.b). The phage isolates showed different plaques according to *Pcc* isolates. Based on morphological characters of plaques, twenty-three isolates were obtained and made circular, clear plaques, clear plaques with or without halo and ranged between 1 mm to 5 mm in diameter. Phage isolates were signed as Ph1 to Ph23. Phage particles were purified by a syringe filter of 0.4 nm and propagated.

Delusion endpoint (DEP) for phages ranged between 10^{-1} to 10^{-9}. Results of phage stability show that all phages remain stable at 4°C for one month and for three weeks at 30°C and 40°C, but some phages lose their infectivity above three weeks at 30°C and 40°C.

The susceptibility of *Pcc* isolates and other bacterial hosts for the phage isolates were examined using a spot test. It was found that *Pcc*1 had sensitivity to all phage isolates, while *Pcc* isolates 2, 3 and 4 had sensitivity to some phage isolates. Some bacteriophages specific to *Pcc* have a broad host range and can infect other genera besides *Pcc* isolates. Results show that *Pseudomonas* sp. and *Xanthomonas* sp. had sensitivity to *Pcc* 23, 20, 9, 10, 7, 5, while three isolates from *Pcc* phages infect pseudomonas sp. and two *Pcc* phage isolates lysis *Xanthomonas* sp. and *Pcc* isolates. In otherwise, *Serratia liquefaciens* group, *Bacillus* sp. and *E. coli* were resistant to all phage isolates.

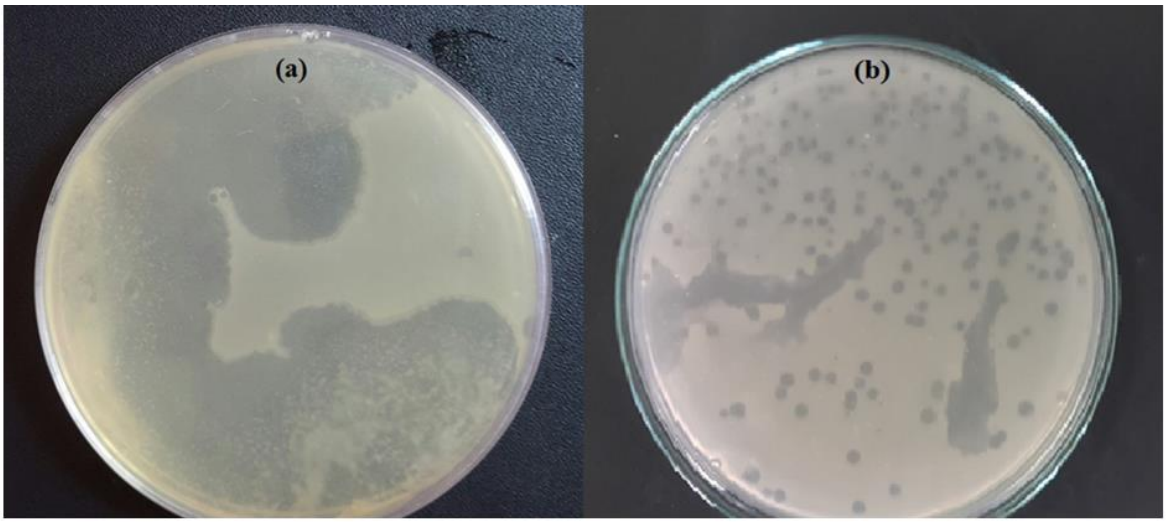

Fig. 1. Techniques for *Pectobacterium carotovorum* phage' isolates, **a)** spot test; **b)** plaque assay.

Electron Microscope:

Transmission Electron microscopy (TEM) reveals that isolated phage particles had long heads, contractile, noncontractile and short tails (Fig.2). The bacteriophages resemble those of the Podoviridae, Myoviridae and Siphoviridae families (Caudovirales order) according to the International Committee on Taxonomy of viruses (ICTV).

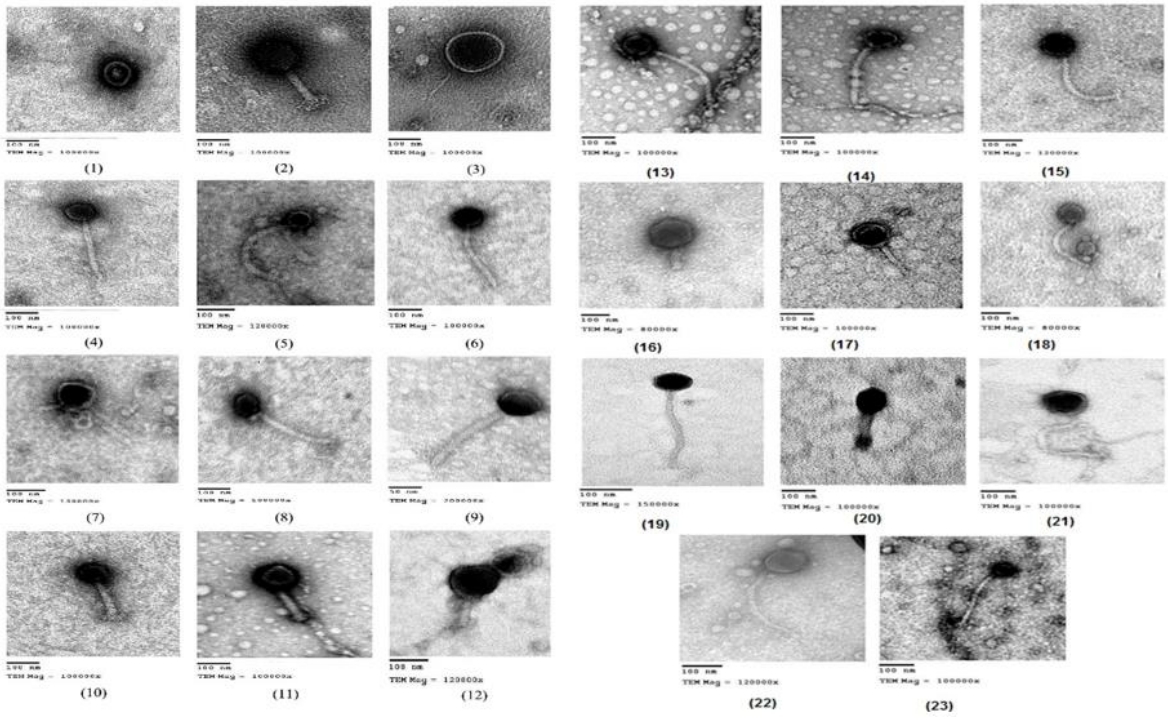

Fig. 2. Electron micrograph for specific bacteriophages of *Pectobacterium carotovorum*

The Ant virulence Activity of Tested Phages:

- **Biofilm formation of *Pcc* isolates:**

Results presented in Table (1) and graphically illustrated in Fig. (3) show that 4 of 20 *Pcc* isolates exhibit biofilm formation where *Pcc*1, *Pcc*2 and *Pcc*4 show strong biofilm formation at about 1.1, 1.4 and 0.9, respectively. Meanwhile, *Pcc*3 showed moderate biofilm formation about 0.6.

Table 1: Biofilm formation assay of *Pcc* isolates using Tissue culture plate method by ELISA reader.

	Bacterial isolates			
	***Pcc*1**	***Pcc*2**	***Pcc*3**	***Pcc*4**
Negative control	0.11	0.11	0.11	0.11
Biofilm (OD_{570nm})	1.4	1.1	0.6	0.9
Biofilm production	Strong	Strong	Moderate	Strong

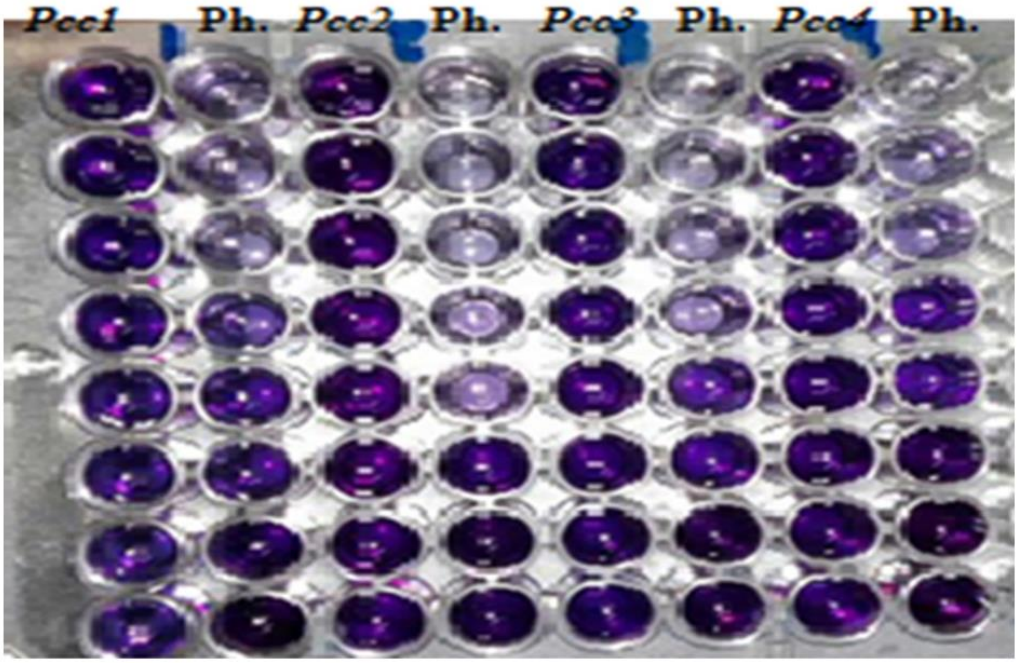

Fig. 3. Tissue culture plate indicating biofilm formation of *Pcc* isolates and their inhibition with bacteriophages (Ph.) by ELISA reader.

- **Biofilm inhibition activity by Phages:**

Twenty-three *Pcc* phage isolates were investigated for their potential effect to prevent biofilm formation against the tested bacterial isolates *ex. Pcc*1, *Pcc*2, *Pcc*3 and *Pcc*4.

The antibiofilm activity of all tested twenty-three phage mixtures against *Pcc*1 biofilm was studied. It was found that bacteriophages potential inhibited biofilm formation of *Pcc* isolates at concentrations (Sub dilution endpoint) 5x 10^9, 15x10^{11}, 15x 10^7 and 6x 10^8 (PFU/ml), while the antibiofilm formation effect in these concentrations was 0.02, 0.21, 0.11 and 0.02 (O.D.), with biofilm reduction percentages were 98.5%, 81%, 81.6% and 97%, respectively for *Pcc*1, *Pcc*2, *Pcc*3 and *Pcc*4, respectively.

Table 2. Antibiofilm formations assay against *Pcc* isolates by specific phages.

	Sub Dilution End Points (DEP)			
	***Pcc*1 (5×10^9)**	***Pcc*2 (15×10^{11})**	***Pcc*3 (15×10^7)**	***Pcc*4 (6×10^8)**
Biofilm formation	0.02	0.21	0.11	0.02
Biofilm reduction (%)	98.5	81.0	81.6	97.0

Phage Therapy for Bacterial Soft Rot Disease on Potato Tubers:

- **Potato tuber assay (Virulence assay):**

Whole potato tubers were used to evaluate the efficiency of *Pcc* phage isolates against *Pcc*1, *Pcc*2, *Pcc*3 and *Pcc*4 and to protect potato tubers from soft rot infection. Spraying of potato tubers with both phage cocktail and bacterial mixture showed a reduction in disease severity and soft rot. Then again, spraying with formulated phages {phage cocktail, corn flour (0.5%) and sucrose (5%)} increased the phage longevity on potato tubers and showed a remarkable decrease in disease severity and soft rot area. In addition, the concentration of phages in formulated form remained at a high value for more than three weeks compared to unformulated phages. Twenty-three isolates of *Pcc* phages were tested in whole potato tubers for their potential against soft rot disease caused by *Pcc*. The results show that up to 90% and 100% of potato tubers inoculated with four *Pcc* isolates show distinctive soft rot with

disease severity (DS%) about 98, 75, 70 and 72.7% with a diameter of rot about 5.2 4.3, 3.8 and 4 cm with percentage (%R) 92.9, 81, 73 and 72.7% for *Pcc*1 *Pcc*2 *Pcc*3 and *Pcc*4, respectively. When potato tubers were sprayed with phage cocktail and *Pcc*, it was found that 70 to 80% of potato tubers were free from soft rot symptoms and the DS % in infected tubers decreased to 7%, 15, 17.5 and 15%, whereas the diameter of rot decreased to 0.5, 0.9, 0.8 0.7cm and with percentage 9, 15.5, 13.7 and 12%. When potato tubers were sprayed with formulated phages and *Pcc*, it was found that 70 to 80% of tubers were free from soft rot symptoms and the disease severity in infected tubers reduced to 5, 10, 15 and 13%. On the other hand, the diameter of the rotting decreased to 0.4, 0.8, 0.6 and 0.5 cm with a percentage of 7, 13.7, 11.5 and 9.4 as shown in Figures 4, 5 and 6.

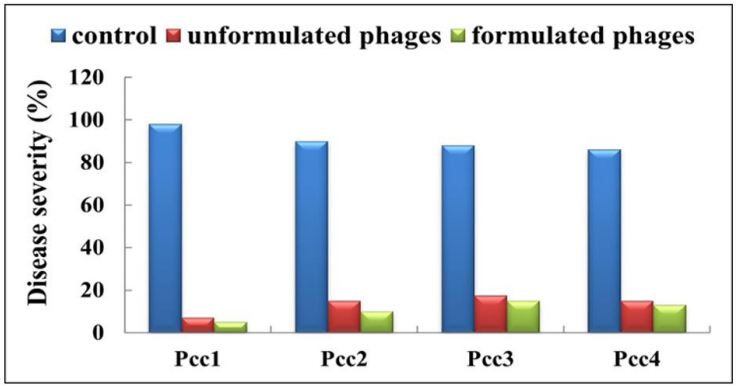

Fig. 4. Disease severity of soft rot symptoms on potato tubers sprayed with *Pcc* isolates, unformulated phages and formulated phages.

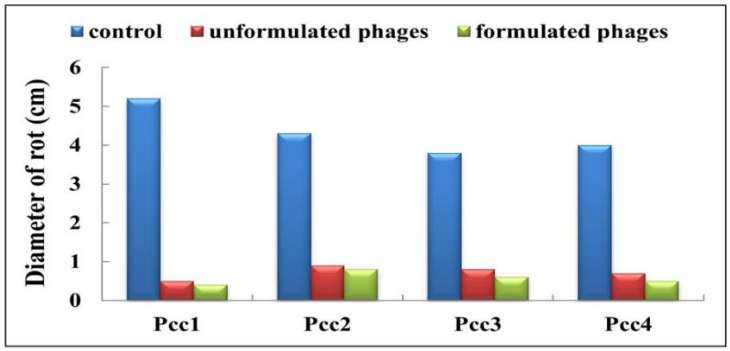

Fig. 5. Diameter of soft rot on potato tubers sprayed with four *Pcc* isolates, unformulated phages and formulated phages.

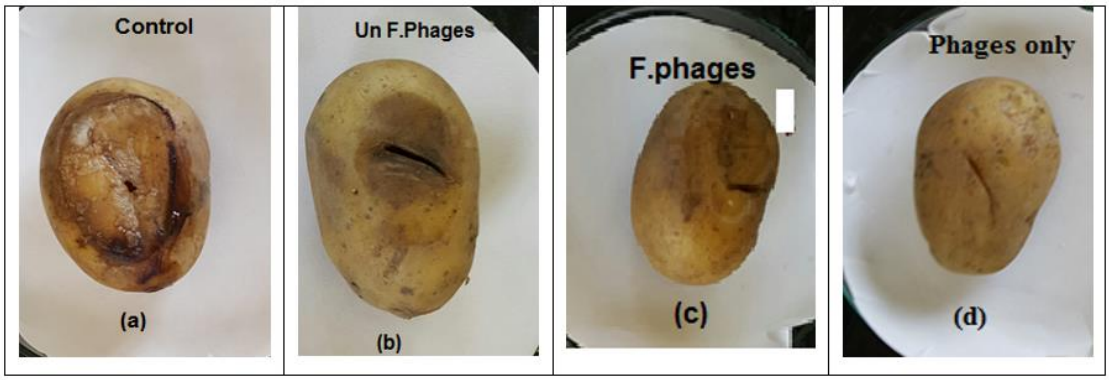

Fig. 6: Photograph showing phage therapy control of soft rot disease on potato tubers. fig. (a): indicates infected potato with Pcc only, fig. (b): treated tuber with unformulated phages, fig. (c): treated tuber with formulated phages.

- **Determination of Phage Titer:**

Soft rot disease causes a great loss in potato yield. All tubers were weighed before and after the experiment and the total loss of tuber tissue caused by *Pcc* isolates were recorded. The results show that the weight of decaying potato tissues due to the infection with *Pcc1* was 52, 47, 45 and 40 g with a percentage of 90.4, 88.6, 81.8 and 80%, respectively. The weight of decaying potato tissues was decreased to 2, 3.02, 4.8, and 2.5 g with a percentage of 4, 5.7, 9.2 and 5% due to spraying tubers with phage cocktail and *Pcc*, respectively. Meanwhile, spraying with formulated phages decreased the decaying potato tissues to 1.5, 2, 1.2 and 1.5 g with a percentage of 3, 4, 2.22 and 3% as shown in Fig. (7).

The titer of unformulated phages was about 1×10^5, 0.9×10^4, 0.3×10^4 and 0.6×10^3 PFU/g. While titer of formulated phages with corn flour and sucrose was about 4×10^{10}, 2×10^9, $5\times10^7$and 2×10^7 for *Pcc*1, *Pcc*2, *Pcc*3 and *Pcc*4, respectively. Formulated phage stability at room temperature was studied for five weeks and examined daily by spot test. It was found that all phage isolates remain stable at room temperature for up to five weeks without losing their activity in the lysis of their host.

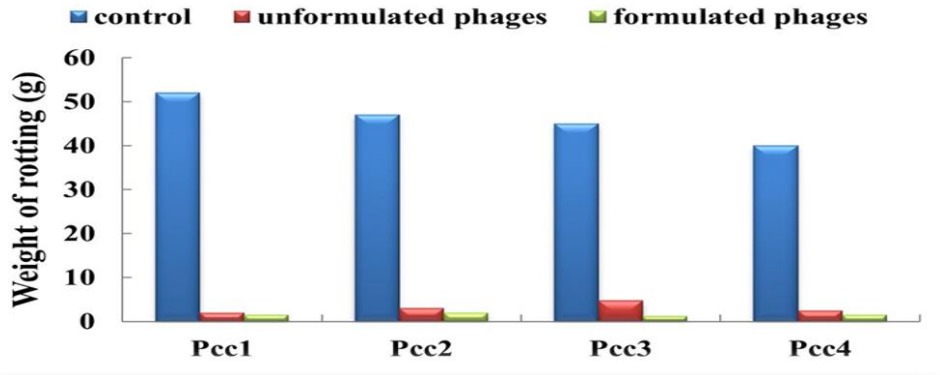

Fig. 7: Histogram showing the weight of rotting in potato tubers sprayed with four bacterial isolates, unformulated phages and formulated *in vitro*.

DISCUSSION

In Egypt, the bacterial soft rot disease *Pectobacterium carotovorum subsp. carotovorum* (*Pcc*) has been a major concern. The disease symptoms in potato storage and open field can show considerable variation at the most distractive where temperatures are moderate and abundant inoculum is available (Kucharek, 1994 and Hassan,2017). It is a key causative factor of soft rot disease on various host plants such as potato, pepper, lettuce, cabbage, and carrot (Ivanović *et al*., 2009). Morphological and biochemical tests revealed that the characteristics of colonies were similar to the standard *Pcc* according to Bergy's Manual of Systematic Bacteriology (Brenner *et al*., 2007; De Vos *et al.*, 2009 and Hassan,2017). It was found that *Pcc* belonged to the Enterobacteriaceae family that possessed circular, creamy, convex, smooth and entire margin, gram-negative, rod-shaped, none forming spores, motile with peritrichous flagella and cannot produce pigments. They grow and form pits on CVP media, creamy colored colonies with no fluorescent pigment on King B medium and produce small and dark red colonies on raffinose medium, the results of the present study are in accordance with the finding obtained by (Kettani-Halabi *et al*., 2013 and Mohamed & Selman, 2013). Biochemical tests on *Pcc* isolates gave positive reactions for KOH, pectinase, catalase, methyl red, voges proskaur, casein and gelatin hydrolysis, H_2S production, Citrate and acid production from glucose, sucrose, fructose, lactose, cellobiose and raffinose tests. Conversely, all *Pcc* isolates can grow at 37°C, 5% and 7% NaCl, but all isolates gave negative reactions for oxidase, indole, urease, starch

hydrolysis, and acid production from maltose tests these results are compatible with (Mohamed and Selman, 2013 and Nazerian *et al*., 2013). The biochemical results were confirmed by the API system that revealed that the tested isolates belong to *Pectobacterium carotovorum*, these results are compatible with (Mohamed and seleman, 2013 and Hassan,2017).

Phytopathogens that infect important agricultural plants are able to reduce plant growth as well as crop yield subsequently. Currently, these phytopathogens are controlled through management strategies. Amongst the important ones is the application of specific bacteriophages, small viruses that exclusively destroy bacteria, providing an even more targeting approach. Typically, phages that concentrate on the bacterial pathogens were isolated from different sources and characterized to ascertain their own features required for implementation as biocontrol agents. Furthermore, appropriate design and provision for affected plants are required for ensuring the phages survive and do not possess a deleterious impact on the plant (Frampton *et al*., 2012). Bacteriophages are natural components of the biosphere, which can be readily isolated from everywhere where bacteria are located, including soil, water, plants, sewage, animals and the human body (Vinod *et al*., 2006). Therefore, in the present study twenty-three phage isolates specific for *Pcc* have been isolated from different sources such as rotted potato tubers, cultivated soil and sewage water samples and have been used successfully as a therapeutic agent for the elimination of bacteria.

In these investigations, twenty-three phages specific for four *Pcc* isolates with different concentrations ranged from 10^2 to 10^7 PFU/ml. Similarly, Ravensdalea *et al*. (2007) and Adriaenssens *et al*. (2012) isolated phages against *Erwinia carotovora* subsp. *carotovora*, *Erwinia carotovora* subsp. *atroseptica* from various sources, such as soil, rotting potatoes, carrots and river water, they found produced small clear plaques of different sizes through all phage isolates. The existing results are in accord with the study of (Lim *et al*. 2015, and Eugenia *et al*.,2021) who isolated bacteriophage specific for *P. carotovorum* subsp. *carotovorum* from soil samples that have been gathered from Chinese cabbage fields in South Korea. Similarly, two lytic bacteriophages of *Pectobacterium carotovorum* subsp. *carotovorum, P. wasabiae* and *Dickeya solani* strains were extracted from two potato fields in central Poland (Czajkowskii *et al*., 2015).

All isolated phages specific for *Pcc* isolates (*Pcc*1 to *Pcc*23) from the tested samples and formed circular, clear plaques, clear plaques with/without halo and ranging from 1 mm to 5 mm in diameter. These results agree with Jee *et al*., (2012) who found that soil samples showed bacteriophage with different sizes of plaques on solid media (largest clear plaque, medium sizes and smallest plaques with the majority of *Pcc* strains) (Czajkowski *et al*., 2015 and Mohamed *et al*., 2016). Dilution End Point (DEP) results proved that DEP for phages ranged from 10^{-1} to 10^{-9}. The present findings are in agreement with the study of Mohamed *et al*., (2016) who found that the DEP for phage specific to both *Pseudomonas stutzeri* and *Bacillus pumilus* strains in the two-fold dilutions, *i.e.*, 1/2, 1/4, 1/8, 1/16 and 1/32 of their enriched phage solutions. Usually, bacteriophage has the ability to kill merely a limited array of the bacterial strains (Koskella *et al.,* 2013 and Eugenia *et al.*,2021). This narrow specificity may hinder the property value of bacteriophages in agriculture, particularly in situations wherein one or more closely related pathogen exists.

Susceptibility of *Pcc* isolates and other bacterial hosts for the phage isolates that were examined by spot test revealed that. *Pcc*1 had sensitivity to all phage isolates, while *Pcc* isolates 2 had sensitivity to nineteen phage isolates, but resistant to four phage isolates whereas *Pcc*3 had sensitivity to fourteen phage isolates, but resistant to nine phage isolates. Also, *Pcc*4 had sensitivity to fourteen phage isolates but was resistant to four phage isolates.

It was found that some bacteriophages specific to *Pcc* have a wide host range and can infect other genera besides *Pcc* isolates. Results showed that *Pseudomonas* spp. and *Xanthomonas* spp. had sensitivity to *Pcc* 23, 20, 9, 10, 7 and 5 while *Pcc*2, *Pcc*13 and *Pcc*22 infect pseudomonas spp. and *Pcc*15, *Pcc*16 lysis *Xanthomonas* and *Pcc* isolates. Similarly, (Czajkowski *et al*., 2015 and Eugenia *et al*.,2021) characterized two lytic bacteriophages ΦPD10.3 and ΦPD23 infecting stains of *Pectobacterium carotovorum* subsp. *carotovorum*, *P. wasabiae* and *Dickeya solani* which were collected from two potato fields in central Poland. In otherwise, *Serratia liquefaciens* group, *Bacillus* sp. and *E. coli* were resistant to all phage isolates. Similarly, Phage ϕ MI appears to have a very narrow host range that is very specific to *Eca*, and has no effect on *Pcc*, these results are in agreement with (Toth *et al*., 1997). Phage stability at 4ºC, room temperature 30ºC and 40ºC were studied for four weeks and examined daily by spot test. It was found that all phage isolates remain stable at 4ºC for one month and for three weeks at 30ºC and 40ºC but some phages lost their infectivity above three weeks at 30ºC and 40ºC. These results are in agreement with (Feisal *et al*., 2013) who found that Salmonella phage (St-1) survived for 7 days at 4, 25, 37, 42 and -20°C, the virus remained infectious without any decrease in its infectivity up to 7 days. Similarly, Mohamed *et al*., (2016) detected phages specific for *Bacillus* and *Pseudomonas* for 2, 4, 6, 8, 10-, 12-, 14- and 16-weeks post storage at 4°C. The morphological characteristics of phage isolate revealed by the Transmission Electron Microscope demonstrated different structural dimensions and features. In these investigations, phage particles that are specific for *Pcc* belong to caudovirales that contain Podoviridae, Siphovirdae and Myoviridae families. Tailed phages constitute the order Caudovirales with three families, characterized by long contractile and noncontractile tails or short tails and named respectively *Myoviridae, Siphoviridae,* and *Podoviridae* that have double-stranded DNA (Akreman, 2011).

It was found that phage particles that are specific for *Pcc* belong to Caudovirales that contain Podoviridae, Siphovirdae and Myoviridae families. Four Phage particles belong to the Podoviridae family that possess a head with a diameter ranging between 57.1 to 190.9 nm, and short tail with a length ranging between 78.5 to 100 nm and a diameter from 10.7 to 36.6 nm. Similarly, TMV examination revealed that the PP1 phage that is specific for *Pcc* is one of the Podoviridae family of the order Caudovirales, which exhibit icosahedral heads with a diameter of ~60 nm, and short non-contractile tail

In the same regard, five phage particles have a long contractile tail and isometric head with diameters ranging between 64.2 to 107.1 nm and lengths ranging between 67.8 to 114.2 nm. While the diameter of the tail ranged between 14.2 to 35.7 nm and the length ranged from 135.7 to 178.5 nm and they are belonging to Myoviridae family. These results were matched with those of Pickard *et al*. (2010) who reported that LIMEstone1 phage specific for *Dickeya solani* which causing soft rot on potato tubers a member of the Myoviridae by transmission electron microscopy with an icosahedral head with 91.4 nm and tail dimensions of 113.8, 617 nm, its morphology is similar to *Salmonella* phage ViI. Similarly, two lytic phages ΦPD10.3 and ΦPD23.1 specific for *Pectobacterium carotovorum* subsp. *carotovorum*, *P. wasabiae* and *Dickeya solani* have morphological features compared with other members of the Myoviridae family and the Caudovirales order, with a head diameter of 85 and 86 nm and length of tails of 117 and 121 nm, respectively (Czajkowski *et al*., 2015).

Phage PM2 specific for Pcc has a head diameter of 90 nm and a contractile tail length of 90 nm, according to transmission electron microscopy pictures, and it belongs to the Myoviridae family in the order Caudovirales (Lim *et al*., 2015). This phage has a larger head and a shorter tail than Pcc phage PM1, which has a head diameter of 55 nm and a contractile tail length of 120 nm (Lim *et al*., 2014).

According to ICTMV (2000) Myoviridae family has phages with a wide host range and can infect members of the Enterobacteriaceae family as *Pcc* (Luhtanen *et al*., 2007). On the other hand, fourteen sperm-shaped phage particles have an isometric head with a diameter ranging between 40 to 92.8 nm and a length between 41.1 to 100 nm with long non-contractile tail with a diameter of 11.7 to 21.4 nm and a length between 111.7 to 407 nm, which they belong to Siphoviridae. These findings are consistence with the finding of Lee *et al*. (2012) who reported that morphological analysis of phage My1 specific for *Pcc* revealed that is a T5-like bacteriophage and belongs to the family Siphoviridae. Similar results were obtained by Kishko *et al*. (1983) who announced that the temperate phage 59 particles specific for *Erwinia carotovora* has an icosahedral head and a long noncontractile tail belonging to the Siphoviridae family. Similar results were reported by some investigators. Mohamed *et al*. (2016) isolated phage specific to soft rot bacteria from soil and soft rot-diseased potato tubers. Electron microscopy of phage suspensions showed the presence of sperm shapes like virus particles with a long tail and icosahedral head and belonging to Siphoviridae family. Keel *et al*. (2002) reported that the Phi GP100 phage with an icosahedral head, and a stubby tail. Analysis of the morphology of phage specific to *Pseudomonas tolaasii* with an electron microscope showed that phi Pto-bp6g comprises an icosahedral head and a long tail, which was categorized as the family of Siphoviridae (Thi *et al*., 2012). In the last decade, the continued emergence of antibiotic-resistant bacteria has led to increasing interest in phage therapy. Phages have been examined as potential agents for biofilm production and soft rot disease control.

Bacterial biofilm may be considered an important virulent factor for several plant pathogenic bacteria such as *Pectobacterium carotovorum*. In this study, three *Pcc* isolates exhibited biofilm formation. *Pcc*1, *Pcc*2 and *Pcc*4 indicated great biofilm formation at about 1.1, 1.4 and 0.9, respectively. While *Pcc*3 showed moderate biofilm formation about 0.6, the current results are in harmony with the study (Hossain and Tsuyumu, 2006).

Pathogenicity and biofilm formation which are considered virulent factors for *Pcc* regulated by autoinducers called cell-cell communication (QS) that are established on small signal molecules exhibiting a cell density-dependent activity affecting gene regulation in Prokaryotes. Bacteria may sense an increased cell density by accumulating these autoinducers both within and outside the cell, allowing them to change their gene expression to coordinate actions that need high cell densities (Castillo-Juárez *et al*., 2015 and Wu *et al*., 2015). Among those autoinducers are acyl-homoserine lactones (AHL) in Gram-negative bacteria, short peptide signals in Gram-positive bacteria, and furan molecules known as autoinducer-2 (AI-2) in both groups (Du *et al*., 2014; Brackman and Coenye, 2015). The use of bacteriophage treatment to lyse bacteria in biofilms has attracted growing interest.

In this study, it was found that bacteriophages potential inhibited biofilm formation of *Pcc* isolates at concentrations (Sub dilution endpoint) $5x\ 10^9$, $15x10^{11}$, $15x\ 10^7$ and $6x\ 10^8$ (PFU/ml), while the antibiofilm formation effect in these concentrations were 0.02, 0.21, 0.11 and 0.02 (O.D.), with biofilm reduction percentages were 98.5%, 81%, 81.6% and 97%, respectively for *Pcc*1, *Pcc*2, *Pcc*3 and *Pcc*4, respectively. Our findings are in agreement with the study of Pei and Lamas-Samanamud (2014) and El dougdoug (2016). They found that the inhibition percentage of biofilm formed by *Staphylococcus aureus* ranged from 54.55% to 95.45% by applying mixtures of different concentrations of phages.

Similarly, Meng *et al*. (2011) determined that the biofilms produced by *Streptococcus suis*, specifically strains SS2-4 and SS2-H, might be dissolved by a bacteriophage lysin, designated LySMP with >80% removal. They reported that engineering bacteriophage to express a biofilm-degrading enzyme during infection is one possible solution for avoiding bacterial resistance. Corbin *et al*. (2001) reported that phage T4 can infect and replicate within *E. coli* biofilms and disrupt biofilm topography by killing

bacterial cells. In this respect, Lytic activity of recombinant bacteriophage φ11 and φ12 endolysins on whole cells and biofilms of *Staphylococcus aureus* has been reported (Sass and Bierbaum, 2007).

Interfering with bacterial cell-cell communication, which is required for biofilm formation, is an appealing and new technique for preventing and inhibiting biofilm formation. Targeting the synthesis, recognition, or transport of QS can generally be used to disrupt bacterial cell-cell communication (quorum quenching, QQ). Furthermore, antagonistic small molecules can be used to degrade or change the relevant signalling molecules, or to interfere with signal perception. Familiar naturally occurring examples for QQ proteins are (i) AHL-lactonases hydrolyzing the ester bond in the homoserine lactone (HL) ring to disable the signaling molecule (Chen *et al*., 2013), (ii) AHL-acylases inactivating AHL signals by slashing its amide bond causing in the analogous fatty acids and HL which are not efficient as signals (Kalia *et al*., 2011), (iii) AHL-oxidoreductases lessening the 3-oxo group of AHLs to create comparable 3-hydroxy byproducts (Bijtenhoorn *et al.*, 2011 and Lord *et al*., 2014). In contrast to diverse AHL-quenching processes and substances, only a few AI-2 interfering mechanisms have been thoroughly investigated. These quenching mechanisms are primarily based on S-ribosyl-homocysteine and transition state analogues interfering with AI-2 synthesis (Widmer *et al.*, 2007), or antagonistic small molecules as shown in V. harveyi and E. coli (Roy *et al*., 2013 and Yadav *et al*., 2014). In this regard, these findings suggest that phage-encoded lytic proteins such as endolysins and virion-associated peptidoglycan hydrolases (VAPGHs) have been tested as antimicrobial agents against pathogens, similar to how Pcc and other phage-encoded proteins with polysaccharide depolymerase activity have been tested as antibiofilm agents (Gutiérrez *et al.*, 2015 and Gutiérrez *et al*., 2016). As a result, bacteriophages aren't just bacterial killers; they're also a source of antimicrobial phage-derived proteins that can be used to combat pathogenic bacteria.

The addition of phage cocktail compared with the bacterial inoculum on potato tubers showed a remarkable decrease in disease severity, the diameter of the rotting area, the percentage of rotting, the number of rotten tubers and the amount of rotted tissue in the diseased tubers. One of the main difficulties in applying bacteriophages to control phytopathogenic bacteria in agriculture is their high specificity *i.e* infecting only the specific strains of the focus on bacterial species (Adriaenssens *et a*l., 2012). This problem has been moderately defeated by using bacteriophage cocktails consisting of several phage mixtures differing in host specificity to maximize the host range covered (García *et al*., 2008 and Chan *et al*., 2013).

The extensive characterization of *Pcc* phages revealed their suitability for phage therapy. Phage cocktail containing phage mixture (10^{11}PFU/ml) applied to healthy potato tuber cv. spunta after inoculation of *Pcc* (10^8 CFU/ml) isolates. Healthy potato tubers were inoculated with *Pcc* isolates separately then inoculated with unformulated phages and formulated phages (phage cocktail with corn flour and 5% sucrose). Potato tubers inoculated with *Pcc* only showed intensive soft rot symptoms and loss in potato yield production. Disease severity reaches 98, 90, 88, and 86%, and diameter of rot 5.2, 4.3, 3.8, and 4cm with a percentage of 92.9, 81, 73 and 72.7 % for *Pcc*1, *Pcc*2, *Pcc*3 and *Pcc*4, respectively. After the application of the phage cocktail, a remarkable decrease in soft rot was noticed. Disease severity decreased to reach about 7, 15, 17.5 and15 % and the diameter of rot decreased to 0.5, 0.9, 0.8 and 0.7 cm with a percentage of 9, 15.5, 13.7 and 12 for *Pcc*1, *Pcc*2, *Pcc*3 and *Pcc*4, respectively. The bacteriophages reduce soft rot infection caused by *Pcc* by at least 91, 75, 70 and 71%. These findings are consistence with the finding of (Czajkowski *et al*., 2015) who used the bacteriophages ϕPD10.3 and ϕPD23.1 individually and when applied together were able to reproduce and significantly reduce soft rot infections caused by

different strains of *P. carotovorum* subsp. *carotovorum*, *P. wasabiae* and *D. solani* by at least 80% to 95% in comparison to controls inoculated with a mixture of bacteria only.

One of the greatest challenges in using phages for plant disease control is their extremely short residual activity in the phyllosphere several studies indicated that phage populations can drop to undetected levels hours after applications (Iriarte, *et al*., 2007). Field and laboratory studies have demonstrated that bacteriophages are inactivated when exposed to high temperatures, moisture, sunlight, and rain (Ignoffo and Garcia, 1992). Ultraviolet-A and ultraviolet-B spectrum (280-400 nm) of sunlight is the most destructive environmental factor (Ignoffo and Garcia, 1994). Also, the presence of free water could significantly increase the extent of virus inactivation by sunlight (Ignoffo and Garcia, 1992). A decrease in the number of phages resulted in less suppression of soft rotting.

Phage therapy can only work when a sufficiently large number of phages are added. There is a real need to keep the titer of phage in high value by increasing the residual activity of the phages which can lead to increased efficacy of phage treatments and to a more convenient application schedule. In this investigation, potato tubers sprayed with Phage cocktail, corn flour and sucrose increased the longevity of phages on potato for more than three weeks and also showed a remarkable decrease in soft rot symptoms and disease severity that decrease to 5, 10, 15, and 13% and the diameter of rot decrease to 0.4, 0.8, 0.6 and 0.5 cm with a percentage of 7, 13.7, 11.5 and 9.4 % for *Pcc*1, *Pcc*2, *Pcc*3 and *Pcc*4, respectively. The formulated phages reduce the soft rot infection by at least 93, 80, 73 and 73 % for *Pcc*1, *Pcc*2, *Pcc*3 and *Pcc*4 respectively. Titer of unformulated and formulated phages was calculated in potato tubers after three weeks. It was found that the longevity of unformulated phages decreases after three weeks with a concentration of $1x10^5$, $0.9x10^{4,}$ $0.3x10^4$ and $0.6x10^3$ PFU/g for *Pcc*1, *Pcc*2, *Pcc*3 and *Pcc*4, respectively, but the longevity of formulated phages not affected by storage more than three weeks with a concentration of $4x10^{10}$, $2x10^9$, $5x10^7$ and $2x10^7$ for *Pcc*1, *Pcc*2, *Pcc*3 and *Pcc*4, respectively. It was found that 10^7 and 10^9 PFU/ml compared to the initial concentrations did not decrease the efficacy of disease control. Potato yield increased with phage treatment up to 70% when all tubers were inoculated with *Pcc* isolates. The current findings corroborate those of Balogh (2002), who produced three formulations to increase the lifespan of bacteriophages on plant foliage for the management of tomato bacterial spots. These formulations were (i) PCF (0.5% pregelatinized corn flour (PCPF + 0.5% sucrose), (ii) Casecrete (0.5% Casecrete NH-400, a water-soluble casein protein polymer + 0.5% sucrose + 0.25% PCPF 400)), and (iii) skim milk (0.75% powdered skim milk + 0.5% sucrose). In comparison to non-formulated phage populations, these formulations resulted in a 4,700, 38,500, and 100,000-fold increase in phage populations two days after application. In field testing on tomato, the PCF, Casecrete, and skim milk formulations, as well as the non-formulated phages, all reduced disease severity by 22, 33, 27 and 19 percent, respectively, when compared to the usual copper-mancozeb therapy. In three field studies, the PCF and Casecrete formulations reduced disease severity by 11 and 21%, respectively, when compared to the non-formulated phage. In one field experiment, the skim milk formulation reduced disease severity by 10% relative to the non-formulated phage treatment. In this regard, Tamez-Guerra, *et al*. (2000) reported that lignin and corn flour formulations of *Bacillus thuringiensis* phages showed 35% more activity after simulated rain, and 12% more activity after simulated sunlight than Dipel 2X, a commercially used insecticide containing *Bacillus thuringiensis*. Additionally, the lignin and corn flour-based formulation had significantly higher residual activity than Dipel 2X, 4 days after application. Similarly, El dougdoug (2016) found that the longevity of unformulated or free phages specific to *Staphylococcus* decreased after three weeks with a concentration of $3x\ 10^5$PFU/ml, but longevity of formulated phages was not affected by storage up to one month with a concentration of $2x10^7$ PFU/ml compared with the initial

concentration 3x 10^7. The interpretation of the obtained results is that these substances increase the residual activity of bacteriophages on potato tubers by keeping them in encapsulated form or forming a film that protects phages from unfavorable conditions.

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