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Isolation and Speciation of a New Bacteriophage for Controlling *Pectobacterium atrosepticum*

Elhalag, K.M.

Bacterial Diseases Research Department., Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt

Many trials were performed in this study for isolation and purification of bacteriophage; that has lytic activity against *Pectobacterium atrosepticum,* previously known as *Erwinia carotovora* sub sp. *atroseptica.* This pathogen is a common phytopathogen that causes significant economic losses in Egypt and worldwide. For developing a biocontrol agent for this blackleg or soft rot pathogen of solanaceous plants, a virulent *P. atrosepticum* phage (ϕPaP2) affiliated to the family *Podoviridae* was successfully isolated from soil in the Nile Delta of Egypt. Its morphological, epidemiological characteristics, and lytic activity was included in this study. The phage was isolated and purified following a single plaque isolate approach, then propagated in bacterial cell suspension inoculated by the Multiplicity Optimal Infection (MOI) of the phage. The phage plaque size was 2.0–3.0 mm in diameter. Morphological examination by Electron microscope showed that phage has an icosahedral head of 55-60 nm in diameter and a short tail of 15-20 nm in length that might belong to the family *Podoviridae* order *caudovirales.* The entire head structural protein was approximately 37 kDa in molecular weight, detected by the usual SDS–PAGE technique. The genomic size and genes function of the phage will be studied in a future study. The optimal MOI value of the phage was 1.0 based on the average count of plaque-forming units (PFU). The single-step growth curve showed that the latent period of infection for ϕPaP2 phage was 50 min, while its complete infection cycle was 150 min. The phage has an average burst size of ∼38 virions per bacterial cell.

The *in vitro* stability of phage has shown that it has the ability to retain activity at temperature up to 70ºC but lost its activity at 75ºC or higher, while the optimal temperature of phage activity was at 25ºC. The phage showed optimal activity at pH 7, while lytic stability was maintained at pH range 4-9.

The host range of the ϕPaP2 phage /specificity was tested against 18 isolates; 7 belong to *P. atrosepticum,* 11 were non-

target bacterial isolates, including other bacterial pathogens of potato and some isolated antagonistic bacteria.
The bacteriolytic activity of phage was expressed *In Planta* (Potato tubers cv. Spunta) that displayed 100% inhibition of soft rotting of tissues (maceration) caused by the pathogen after incubation at 25°C for 48 h compared to phage-untreated tubers. The longevity of phage ϕPaP2 was studied in different soil types. In general, the longevity of the phage was found higher in clay soil than sandy soil. The presence of the host bacterium in both clay and sandy soil showed a remarkable increase in the longevity of the phage compared to bacteria- untreated soil. In conclusion, the isolated bacteriophage has had the ability to suppress *P. atrosepticum in vitro* and to protect potato tuber from rotting caused by the pathogenic bacteria in concern.

Keywords: Soft rot; blackleg; bacteriophages; bio-agents

Potatoes in Egypt represent an important economic cash crop. The annual rate of potato exportation was at a steady increase. Potato crops might subject to several diseases that negatively affect the quality and quantity of potato yield. In order to sustain the reputation of Egyptian potato, the management of such potato disease should be applied in an ideal and environmentally friendly way. Bacterial diseases such as potato blackleg and tuber soft rot caused by the pectinolytic bacteria (Pectobacterium and recently Dickeya species) may result in an important loss in local potato production in Egypt (Ashmawy *et al*., 2015). *Pectobacterium* is considered one of the main bacterial agents responsible for the disorder of potato tubers (van der Wolf and De Boer, 2007). It is a pectinolytic Gram-negative bacterium that causes soft rot disease, wilt, or blackleg in many host plants (Lim *et al.*, 2013). *Pectobacterium* affects either growing plants or tuber storage and results in transit problems and drastic effective economic losses (Czajkowski *et al.*, 2015). Accordingly, it is an important disease to be considered in potato production and storage at ambient temperatures.

Pectobacterium is sub-divided into six species and subspecies: *atrosepticum*, *betavasculorum*, *carotovorum* subsp. *brasiliense*, *carotovorum* subsp. *carotovorum*, *carotovorum* subsp. *odoriferum*, and *wasabiae* (Lim *et al.*, 2015). *P. atrosepticum* typically causes blackleg symptoms under cool wet conditions. In Egypt, both *P. carotovorum* and *P atrosepticum* have been previously isolated and considered as the main pathogens causing tuber soft rot and/ or blackleg diseases respectively (Ashmawy *et al*., 2015). *P. atrosepticum* can survive for reasonable time in their host plants and respective soils. Recently, researchers have had renewed their interests in applications of bacteriophages as antibacterial agent, due to their specific potential in lysing and targeting the corresponding host bacteria. The chemicals used

to control such diseases have had limited efficacy beside the variety of problems that include environmental hazards and residual chemicals on crops.

Furthermore, literature review for using of bacteriophages that able to lysis of *Pectobacterium* species has been suggested as a promising tools of controlling the soft rot disease due to their target specificity, economic feasibility, and safety (Jee *et al.*, 2012; Czajkowski *et al.*, 2015). Bacteriophages capable of multiplication within the hosting bacteria, so the number of bacteriophage particles can be increased at target point.

Despite of the large diversity of bacteriophages used to control soft rot diseases, just few research studies have been performed to identify the phages capable of infecting *Pectobacterium* in Egypt. In this context, the main aim of this study is to isolate, purify and characterize a possible new phage that have lytic activity against the soft rot disease in Egypt caused by *P. atrosepticum.* Physical characteristic and structural protein of the phage was conducted in this study. The effect of different temperature and PH regimes on the lytic activity of the phage was also included. The lytic activity of the phage against pathogenic *P. atrosepticum* was studied in *In vitro* and *planta.* The specificity and host range of the phage were studied against variety of bacterial isolates. The longevity of isolated phage was also studied in different soil types.

Materials and Methods

Bacterial cultures and growth conditions, and verification of isolates:

Strains of *P. atrosepticum* used in this study embracing seven virulent strains, were previously isolated in Egypt from diseased potato tubers and stems, with soft rot and blackleg disease (*In press*). Strain MH3c was used for isolation of the phage, while other strains were used for studying the specificity and the host range of the isolated phage. Three strains of *R. solanacearum* (K3, K10 and K16) were also used in this study with other bacterial isolates for studying the specificity of the phage (Elhalag *et al*., 2015). The other bacterial strains were kindly provided by Science and Technology Development Funding (STDF) project No. 2905. These strains include plant pathogenic bacteria and antagonistic bacteria. All bacterial strains used in this study were continuously subcultured on nutrient agar and King's media slants (King *et al*., 1954) for routine testing and maintenance of bacteria (short-term usage), while long preservation was done at -30°C using sterilized glycerol buffer (20%) (Hayward, 2000). Logan's medium (nutrient agar 28.0 g, yeast extract 5.0 g and glucose 5.0 g dissolved in distilled water 1000 ml) was also used for describing the morphology of *P. atrosepticum* on media (Fahy and Hayward 1983). Molecular detection was utilized for confirming all isolates of *P. atrosepticum.*

In this context, DNA was extracted using a simple method described by Sambrook *et al.* (1989) from pure bacterial cultures grown on NA media at 25°C. Specific primers (ECA-1F: CGGCATCATAAAAACACG and ECA-1R: GCACACTTCATCCAGCGA), obtained from Bio Basic, Canada were used for

detection of *P. atrosepticum* using standard PCR assays. Amplification program cycles were carried out using thermal cycler (T- personal- Biometra, (Biometra GmbH, Göttingen, Germany) according to DeBoer and Ward (1995). 25µl of PCR reaction mixture were prepared as follow:

Five µl of the ready PCR master mix (My *Taq*- red DNA polymerase, Bioline, Germany), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 3 µl of the template DNA and 17 µl of ultra-pure water.

PCR products was separated by electrophoresis in a 1.5% agarose gel and stained with Red Safe (Intron, Korea) in TAE buffer. DNA ladder (1kb plus, Invitrogen, Thermo Fisher Scientific, USA) was used in each loaded gel as reference in at least one well. Gel was run on 80 V voltages at 400 mA (8 v/1cm). PCR bands were visualized under UV trans-illumination (at 355 nm).

Soil samples and symptomatic rotting potato tissues:

Different soil samples (16 samples) were collected from cultivated potato fields at various locations of the Nile Delta in Egypt. The field locations were at El-Behira (Sandy soil) and El- Ghrabyia (clay soil) governorates and were used for bacteriophage isolation and studying the duration of survival for the isolated phage. Rotting potato tissues (8 samples) showing typical symptoms of soft rot and blackleg diseases were also used for isolation of bacteriophage.

Isolation, purification, and propagation of the phage:

For phage isolation, samples of 10g soil or rotting tissue were suspended in 100 ml of the king's broth medium previously inoculated with dense *P. atrosepticum* strain (MH3c) with a concentration of 9×10^8 CFU/ml. The inoculated flasks were incubated for 12-18 h in a shaking incubator (SHKE481HP, Thermo-Scientific, USA) at 120 pm at 25°C to release bacteriophages in concern from soil particles or tuber tissue. The suspensions were briefly centrifuged for 10 min at 5000 rpm to remove the bacterial cells. The resulting supernatant after centrifugation was then filtered through a membrane filter (0.22 µm pore size; Steradisc, Kurabo Co. Ltd., Osaka, Japan) using syringe filters. One hundred µl of the suspension were used as aliquots for plaque-forming assay using the same original strain used before. The plaque assay was carried out according to Mihara *et al*. (2016) using king's plates containing 2% agar and overlaid with 0.8% king's soft agar to make double layer agar plates. The plates were incubated at 25°C for 24 or 48 h. The shape, size, and number of plaques were recorded after the incubation time, and plaque formation was examined visually by eye. Spot test was also performed to confirm plaque assay results as follows: *P. atrosepticum* bacterial cells of 10^8 CFU /ml were mixed with melted 0.8% agar (50°C), and the mixture was poured on a 2% solid agar of King's medium to make double layer agar plates. After media solidification, about 5 µl of filtered supernatant containing phage were spotted on the surface of each agar plate. Lysis spots were observed after the incubation period. Isolated

phage obtained from either plaque and spot assay was purified from single plaque isolates and then propagated as follow: a culture of bacterial cells (16-24 h grown in a liter of liquid King's medium and incubated at 25°C with a concentration of 7×10^8 CFU/ml) was diluted 100-fold with 100 ml fresh King's medium in a 500 ml flask.

The phage picked from a single plaque was added at a multiplicity optimal of infection (MOI) of 0.1, 0.5 1.0, and 2.0. After 18 h of incubation, were centrifuged to remove the cells using a refrigerated centrifuge (SIGMA 3-18K, Sigma Laborzentrifugen GmbH, Germany) at 8800 xg (6500 rpm) for 15 min at 4°C. The supernatant was filtered through a 0.2 µm membrane filter (Gelman Sciences, USA), and phage particles were precipitated by centrifugation at 20400 xg (13500 rpm) for 2 h at 4°C and then suspended in SM buffer (50 mM Tris-HCl at pH 7.5, 100 mM NaCl, 10 mM $MgSO_4$, and 0.01% gelatin). The final concentration was approximately 4×10^{11} PFU/ml that was determined using the plaque assay. Purified phages were stored at 4°C for further study.

Bacteriophage particles morphology under transmission electron microscopy:

The suspension of propagated and concentrated phage particles was stained with 2 % uranyl acetate before observation in a JOEL-JM-100-C transmission electron microscope (TEM) (Japan Electron Optics Laboratory Co., Ltd.) according to Yamada *et al*. (2007). Microscopic analyses were performed in the Laboratory of Electron Microscopy at Cairo University Research Park (CURP), Giza, Faculty of Agriculture, Egypt.

Determination of phage structural protein:

The structural proteins of phage were detected using 12% SDS-polyacrylamide gel electrophoresis (PAGE) (BioGmetra, Germany) based on the method described by Laemmli (1970). The whole test was performed at Cairo University Research Park (CURP), Faculty of Agriculture, Giza, Egypt.

The structural phage protein bands were visualized on the gel, and the approximate molecular weights were determined compared to standard protein marker (GangNam-STAIN™ Prestained Protein Ladder, iNtRON Biotechnology, Inc.). The gel was then photographed and reasonably documented using the photo documentation system (Syngene, G: Box, UK).

Detection of optimal multiplicity of infection:

The optimal multiplicity of infection of the isolated phage was determined against the host bacterial cells (*P. atrosepticum*) according to Gasic *et al*. (2011). In this context, 24hrs culture of *P. atrosepticum* was infected with the phage at different (MOI) (0.1, 0.5, 1, and 2). The MOI resulting in the highest phage titer (the highest PFU/ml) was considered optimal. The entire experiment was repeated three times with the same setup, and the results from all repetitions were averaged.

One step growth curve of the phage:

The latent period and burst size for the phage (one-step growth curve) were carried out according to Carlson (1994). *P. atrosepticum* (MH3c) strain was used as a host for the phage. In brief, 24 h bacterial cells suspension grown on King's broth (incubated at 25°C, 0.25 U of OD600) were used to reach a concentration of $4x10^8$ CFU/ml. The optimal MOI (1.0) titer of the phage was added to the bacterial suspension and incubated for 30 min at 25°C for adsorption. After the adsorption period, the suspension was centrifuged, and the pellet was re-suspended in King's broth. The bacterial cells were incubated at 25°C. Samples were taken at intervals up to 5 h (every 30 min following incubation), and the plaque-forming unit was immediately detected (titer determination) according to Mihara *et al*. (2016). The entire experiment was replicated twice. The growth curve that expressed the ratio of PFU per infected cell during each time interval was determined.

Effect of temperature and pH on phage activity under in vitro conditions:

The most important factors that may affect phage activity (stability) were studied. The activity of phage was observed under different temperature degrees (4, 10, 25, 28, 40, 50, 60, 70, 80, and 90ºC) and pH conditions (2, 4, 6, 8, and 10) *in vitro* according to methods described by Jamal *et al*. (2015). 1.5 mL Eppendorf tubes containing a diluted suspension of the bacteriophage were incubated at different temperature regimes for 30 min, then the plaque assay was done as described above as three replicates for each considered temperature.

Phage stability at different pH was conducted as follows: phage stability in King's broth was assayed at pH values 2, 4, 6, 8, and 10, after 3 h of incubation at 25°C, the phage plaques were counted using double-layer agar plate. In each test, stability was assessed as the ratio of the phage particles which survived to the initial number of phages used.

Specificity of phage and host challenge assay:

A host target specificity assay was performed as described by Czajkowski *et al*. (2014) using a range of different bacterial strains. Six *P. atrosepticum* strains and one *P. carotovorum,* isolated previously from symptomatic potato tubers and stems, along with some plant pathogenic bacteria as well as antagonistic bacterial strains isolated from soil by Farag *et al*. (2017)*,* were used (listed in Table 1). The phage specificity and host range were tested either by plaque assay and spot assay according to Armon and Kott (1993). Areas of clear zones under the points of phage application were scored as positive, whereas areas which looked no different to the surrounding untreated lawn were scored as negative. Lysed areas were observed up to 48 h. Control plates (Non-phage inoculated) were used. The entire experiment was repeated twice.

Effect of bacteriophages on controlling tuber maceration caused by P. atrosepticum:

The *In planta* experiment was used to verify the protective effect of ϕPaP2 phage on potato tuber maceration that was performed as described by Czajkowski *et al*.

(2014) using *P. atrosepticum* as a host. Potato cv. Spunta was used for studying the incidence of potato tuber tissue maceration caused by *P. atrosepticum*. Phage concentration was adjusted at 3×10^6 pfu/ mL in sterile water. The *P. atrosepticum* bacterial cells were grown in King's broth medium at 25°C for 16 h. After centrifugation, the cells were resuspended in sterilized water to optimize the cell density to OD = 0.1.and was adjusted to 10^8 CFU/mL. A total of fifteen potato tubers (cv. Spunta) were surface-sterilized with sodium hypochlorite for 10 min, then rinsed with sterile tap water and dried with tissue paper. One well per tuber (cylinder holes with a depth of approximately 5 mm on the surface) were made using a sterile cork borer as five replicate per treatment tubers. Wells were then filled with 50 μL of *P. atrsosepticum* suspension and 5 μL of ϕPaP2 phage at optimal MOI (1.0). The cylinder of removed tissues was replaced then the wound was sealed with sterilized paraffin wax. Five wells in different five tubers were filled only with 50 μL of *P. atrsosepticum* suspension at the same concentration used before (positive control). Another five wells filled with sterile distilled water were used as a negative control. Incubation was made in a dark room at 25ºC and monitored for 7 days. Viable bacterial strains were counted on Logan's medium for each treatment at the end of the experiment and confirmed using conventional PCR using specific primers mentioned before. The protective effect of the phage on the tissue maceration (soft rot symptoms) was calculated as the ratio of the average diameter of rotten potato tissue around wells co-inoculated with *P. atrosepticum* and ϕPaP2 phage to the average diameter of rotten tissue around wells of the positive control. The experiment was replicated twice with the same setup, and the obtained results were averaged per assay.

Duration of survival for isolated phage in clay and sandy soil:

The longevity of the phage was evaluated at room temperature (20-30ºC) in two soil types, (clay and sandy soil); collected from El-Gharbyia and El- Behira governorates respectively. About 200 g of each soil were added to five cups, as replicates, for each soil treatment type. Each cup was inoculated by 50µL of ϕPaP2 phage, and it was mixed well to ensure equal distribution of the phage. Another five cups for each soil type were inoculated with 2mL of 10^9 *P. atrosepticum* suspension, washed out from 24 h old culture, and each cup was amended with 50µL of ϕPaP2 phage. Longevity survival of phage in SM buffer was done by inoculation with the same phage concentration. Moisture content for each cup was fixed, and the cups were weighted daily for moisture loss that was corrected by adding sterilized distilled water till the end of the experiment, all cups were kept at room temperature, and the count of phage particles was monitored in each soil using plaque assay at the days 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 28 days respectively from soil inoculation with the phage. All types of soils were sent to (Soils, water, and environmental research institute, ARC, Giza, Egypt) for physical and chemical analyses.

Statistical analysis:

Data of replicated experiments are presented as means ± standard deviation (SD) of three tested replicates for each treatment. Error bars represent the standard deviation were added to figure (6, 8, and 9). Correlation analyses between the count of phage particles (plaque-forming unit) and different temperatures and pH regimes were performed using software SPSS v16.

Results

Bacterial cultures:

Bacterial isolates of *P. atrosepticum* used in this study showing typical morphology on Logan's medium (Fig. 1).

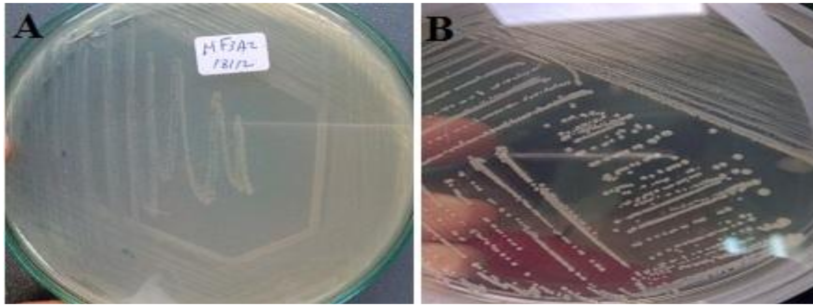

Fig. (1): Morphology of *Pectobacterium atrosepticum* (bacterial growth), Isolated from symptomatic potato plants in Egypt, on King's medium (A) and Logan's medium (B)

The identity of isolates was confirmed by PCR amplification assay using specific primers. ECA primers showed a specific band visualized at 690 bp that confirm the isolated bacteria as *P. atrosepticum* (Fig. 2).

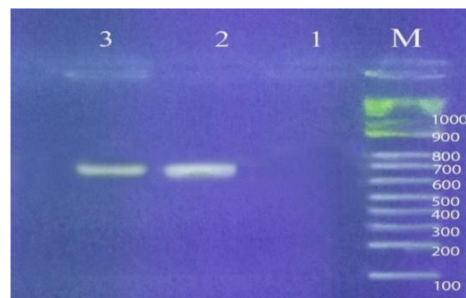

Fig. (2): Agarose gel electrophoresis showing single specific band at 690bp for two bacterial isolates (MF3c and MF8), isolated from Elminufyia governorate in Egypt using a specific primer (ECA primer) for the detection of *Pectobacterium atrosepticum*.

Note: PCR amplification was done according to DeBoer and Ward (1995). M: DNA ladder (1kb), 1: Negative control, 2 and 3: DNA from *P. atrosepticum* (target isolates)

Isolation of P. atrosepticum lytic bacteriophage:

The samples screened for the presence of lytic bacteriophage of *P. atrosepticum* revealed the presence of new lytic phage in soil sample of Elbehira governorates. Among 16 soil samples only one sandy soil, was collected in winter from potato field, the phage was successfully recovered. The activity of phage particles against the host bacteria (*P. atrosepticum*) was observed in both spot test and plaque assay. Clear plaques (Circular in shape) ranged from 2.0 to 3.0 mm in diameter were detected which express the virulent activity of the tested phage (Fig. 3).

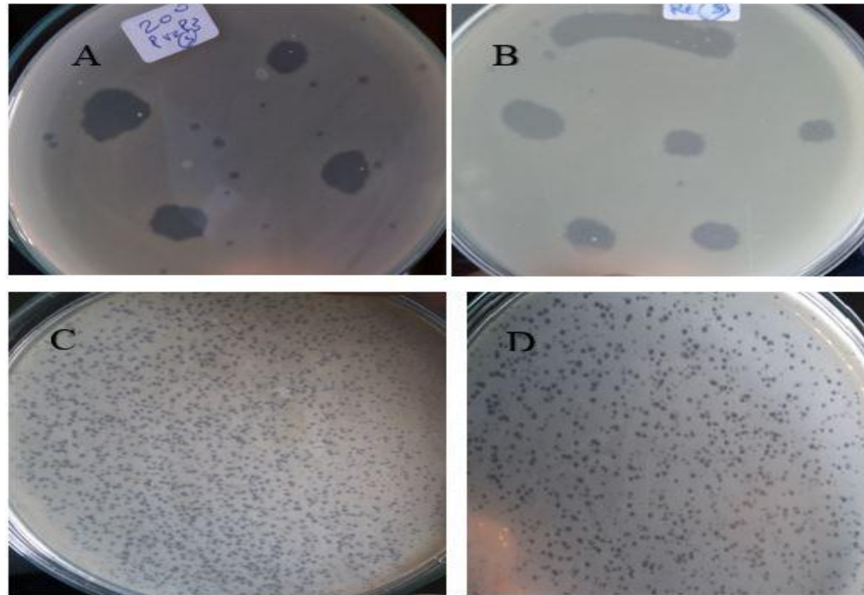

Fig. (3): Spot assay plates showing lytic activity of recovered phage (ϕPaP2) against *P. atrosepticum* (A, B) and plaque assay showing clear plaques 2.0– 3.0 mm in diameter at dilution of (10^{-7}) of the phage titre (C, D).

Note: phage was recovered from El-Behira governorate from potato cultivated field.

The isolated phage was named ϕPaP2 phage. To obtain purified phages, single distinct plaques were picked in SM buffer and then propagated. The phage was never isolated from symptomatic potato tuber tissues using either plaque assay or spot test.

Transmission electron microscopy (TEM):

Transmission electron microscopy analysis performed for ϕPaP2 revealed that the phage belongs to the order Caudovirales and family Podoviridae based on its morphology and presence of the non-enveloped icosahedral head (diameter: 55-60 nm) and non-contractile short tail (length: 15- 20 nm) (Fig. 4).

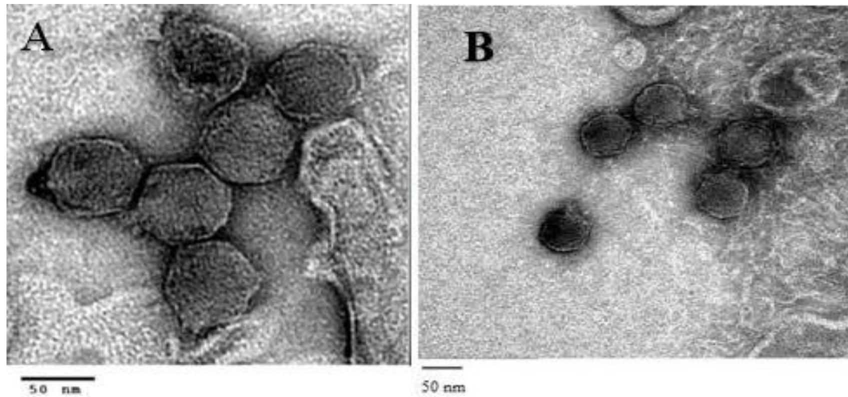

Fig. (4): Two electron micrographs showing morphology of bacteriophage ϕPaP2 isolated from Egyptian soil that infects *P. atrosepticum* (A and B)

Note: Bacteriophage was stained with uranyl acetate before transmission electron microscopy. Bar marker represents 50 nm for A and B.

Structural proteins of ϕPaP2 phage

Phage structural protein detected by SDS-PAGE, showed that ϕPaP2 phage is composed of a major 3 proteins (Fig. 5). The major head protein structure is approximately 37 kDa in molecular weight.

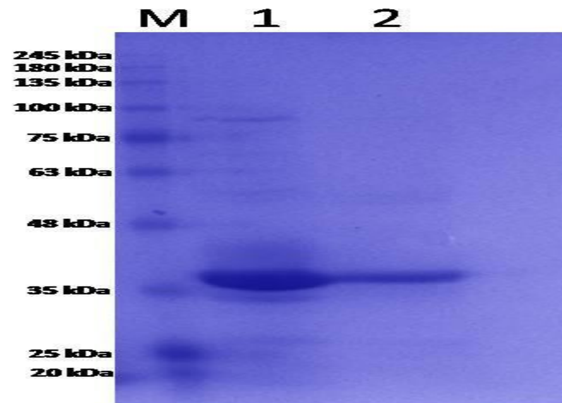

Fig. (5): Structural proteins of phage ϕPaP2, recovered from Egyptian soil against *P. atrosepticum* using SDS-PAGE analysis. Lane M, Protein molecular mass markers, Lane 1 and 2 are duplicate of phage ϕPaP2 proteins with different running volume of 20 and 15 µl respectively.

Optimal multiplicity of infection and in vitro lytic activity of phage

Based on the average count of plaque-forming units (PFU) from three separate determinations, the optimal MOI of phage ϕPaP2 was 1.0. The average count of PFU for the optimal MOI (1.0) was higher than detected in other tested MOI. The potential of the bacteriophage as an antibacterial against *P. atrsosepticum* was studied *in vitro*. The bacterial count (calculated on the basis of the OD 600) showed a remarkable decrease in all tested MOI of the phage compared to pathogen control (phage- untreated) (Fig. 6). The optimal MOI of ϕPaP2 phage showed a decrease in OD of *P. atrsosepticum* that reach 0.2 after 24h of incubation time.

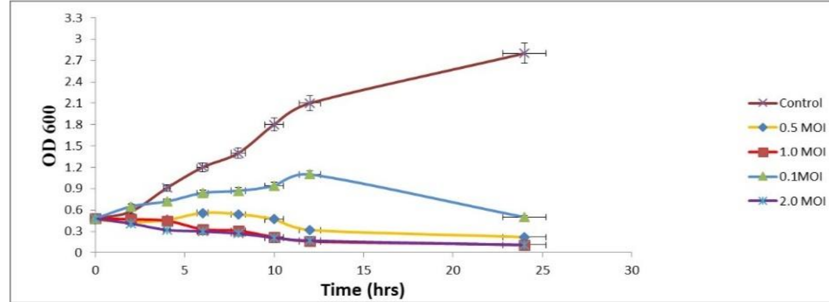

Fig. (6): Growth curve expressed by Optical Density (OD) for *P. atrosepticum* bacterium after infection by phage ϕPaP2, recovered from Egyptian soil (*In vitro* bacteriolytic activity) at different values of MOI: 0, 0.1, 0.5, 1.0 and 2.0.

Note: All values represent means of three run determinations. Error bars represent the standard deviation

One step growth curve of ϕPaP2 phage

The single-step growth curve, showed that the latent period of infection for ϕPaP2 phage was 50 min, while its complete cycle of infection was 150 min. The phage has an average burst size of ~38 virions per bacterial cell (Fig. 7).

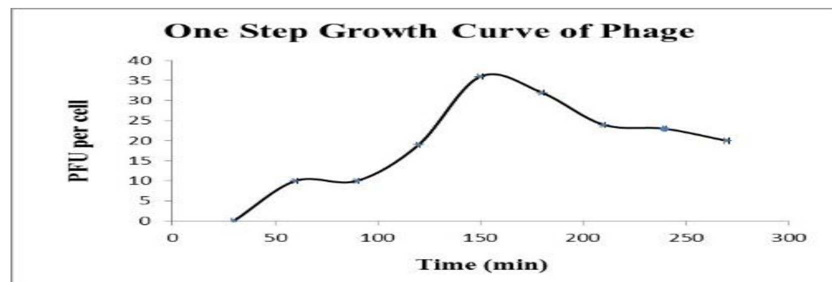

Fig. (7): One-step growth curve for phage ϕPaP2, recovered from Egyptian soil specific for *P. atrosepticum*

Note: Latent time and burst size of phage ϕPaP2 were 50 min and 38 pfu per cell respectively.

Effect of temperature and pH on phage activity in vitro

In general, the stability of bacteriophages under nine different temperature degrees (4, 10, 25, 28, 40, 50, 60, 70, and 80°C) was different as shown in Fig. (8). The optimum temperature for phage activity against *P. atrosepticum* was 25°C. Phage activity was stable at a temperature up to 70°C while it lost activity at a temperature higher than 70°C after being incubated for 30 min.

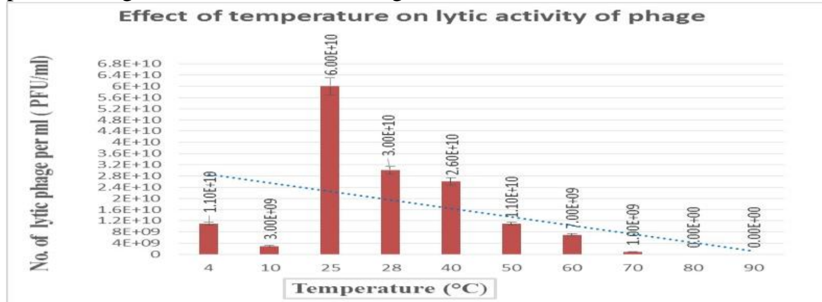

Fig. (8): Bacteriolytic stability of phage ϕPaP2, recovered from Egyptian soil against *P. atrosepticum* at different temperature after 30 min incubation time

Note: The bacteriolytic stability was expressed by Plaque forming unit (PFU) per ml. All values represent means of three run determinations ± SD. Error bars represent ± the standard deviation.

The stability of phage at different pH showed that the phage is stable in neutral pH (pH 6.0-7.0), while the PFU of the phage was decreased both in acidic and basic pHs after incubation (Fig. 9). The phage was not able to survive at pH 2.0 and pH 10 (Fig. 9). The greatest decrease of phage particle numbers was observed at pH 8.0. The correlation between the count of phage particles (phage forming unit) and different temperature degrees and pH regimes was made by correlation analysis using SPSS v16. No significant correlation was found between both temperature or pH value and plaque.

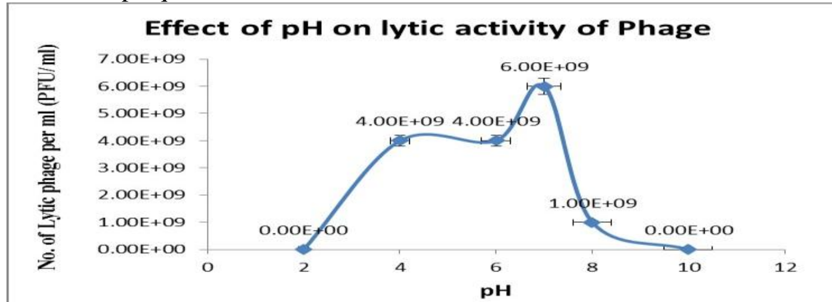

Fig. (9): Bacteriolytic stability of phage ϕPaP2, recovered from Egyptian soil against *P. atrosepticum* at different pH and incubated for 3h at 25ºC.

Note: The bacteriolytic stability was expressed by Plaque forming unit (PFU) per ml. All values represent means of three determinations ± SD. Error bars represent ± the standard deviation.

Specificity and host range of phage

All tested bacterial isolates of *P. atrsoepticum* showed lytic activity caused by the applied ϕPaP2 phage, but the expression was different. The phage showed no affinity against other tested bacterial strains, belonging to gram-positive and gram-negative genera based on either the spot test or plaque assay results. These results indicate that the phage specificity is restricted only to *P. atrosepticum* as a host.

Protective effect of bacteriophages on potato tuber tissue maceration caused by P. atrosepticum

The phage ϕPaP2 was able to 100% decrease in potato tuber maceration caused by *P. atrosepticum* compared to the control tubers inoculated with bacteria only that developed typical maceration after 48 h after incubation (Fig. 10). No soft rot symptoms were detected on potato tuber pre-treated with a phage that was observed for 7 days after the inoculation of bacteria and phage (Fig. 10). No viable bacterial cells could be detected in isolation on logan's medium as well as by PCR amplification in phage-infected tubers. The tuber slices treated with sterilized water showed no rotting symptoms.

Table (1): Specificity and host range of ϕPaP2 phage using different bacterial species

Code	Bacterial strains[a]	Spot test	Plaque assay
MH3c	*Pectobacterium atrosepticum*	+	+
MH1	*Pectobacterium atrosepticum*	+	+
MH2	*Pectobacterium atrosepticum*	+	+
MH6	*Pectobacterium atrosepticum*	+	+
MH8	*Pectobacterium atrosepticum*	+	+
MH10	*Pectobacterium atrosepticum*	+	+
MH11	*Pectobacterium atrosepticum*	+	+
K3	*Ralstonia solanacearum* Phylotype IIa sequevar I	-	-
K10	*Ralstonia solanacearum* Phylotype IIa sequevar I	-	-
K16	*Ralstonia solanacearum* Phylotype IIa sequevar I	-	-
1	*Serratia marcescens*	-	-
2	*Pseudomonas fluorescense*	-	-
3	*Stenotrophomonas maltophilia*	-	-
4	*Bacillus thuringiensis*	-	-
100	*Pectobacterium carotovorum*	-	-
177	*Pseudomonas aeruginosa*	-	-
114	*Enterobacter aerogenes*	-	-
447	*Pseudomonas japonica*	-	-

Sensitivity to ϕPaP2 (+) sensitive, (-) resistant

a) All *P. atrosepticum and R. solanacearum* strains used were previously isolated and identified (*in press* publication and Elhalag *et al*. 2015) respectively, and other bacterial strains used in this study were kindly provided by STDF project No. 2905 in Egypt.

Fig. (10): *In Planta*, pathogenicity test on Spunta potato tubers freedom of soft rot caused by *P. atrosepticum* after treatment with ϕPaP2 phage (A), and photo of bacteriophage free inoculated potato with soft rot symptoms 2 days after inoculation with suspension of 10^8 CFU ml^{-1} of the pathogen (B)

Note: The phage was applied at a concentration of 3×10^6 PFU ml^{-1}. Each hole in the potato tuber was inoculation with 50µl of 24 hrs *P. atrosepticum* bacterial suspension (3×10^8 cfu ml^{-1}) and incubated for 48hrs at 25℃. B: refer to rotting tissues of sealed potato tuber's holes using vaseline after bacterial inoculation.

Duration of survival of ϕPaP2 phage in clay and sandy soil

The duration of survival for the phage was found longer in clay soil than sandy soil. The presence of the pathogenic bacterium in both clay and sandy soil showed a remarkable increase in the longevity of the phage compared to phage- untreated soils. The phage was able to survive for 14 days in non-inoculated sandy soil compared to 22 days of survival in inoculated sandy soil. While it was survived for 20 days in non-inoculated clay soil compared to 28 days of survival in the presence of host in clay soil. SM buffer kept the survival of the phage for only 18 days.

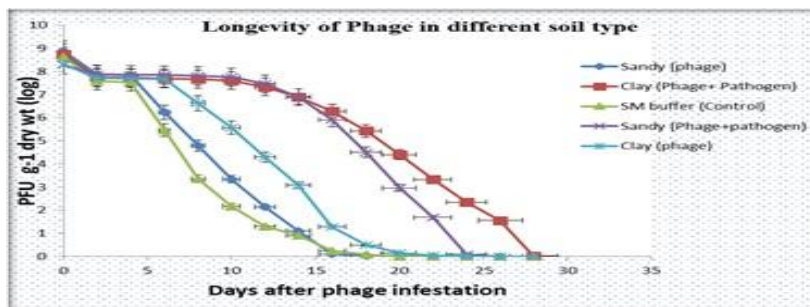

Fig. (11): Survival of phage ϕPaP2, isolated from Egyptian soil against *P. atrosepticum* in different soil types (clay and sandy soil).

Note: The phage was applied to soil and SM buffer at a densities of 6×10^9 PFU ml^{-1}. Error bars represent ± the standard deviation

The physical and chemical properties of clay and sandy soil used was illustrated in Table 2. In general, the NPK content was higher in clay than sandy soil. The cations and anions including: Ca^{++}, Mg^{++}, K^{+}, Cl^{-}, $SO4^{--}$, Na^{+}, $HCO3^{-}$, Fe, Cu, Zn and Mn was in general higher in clay soil than sandy soil.

Table (2): Physical and chemical properties of clay and sandy soil used for studying the survival of phage ϕPaP2, isolated from Egyptian soil against, *P. atrosepticum* in different soil types.

Physical and chemical characteristic	Unit	Clay soil*	Sandy soil
PH	-	7.1	7.3
EC	-	1.08	0.92
SP	-	38.5	23.5
Ca^{++}	ml equivalent/l	1.83	2.6
Mg^{++}	ml equivalent/l	3.4	1.9
K^{+}	ml equivalent/l	0.3	0.14
Cl^{-}	ml equivalent/l	9.1	6.5
SO_4^{**}	ml equivalent/l	1.96	1.08
Na^{+}	ml equivalent/l	2.03	1.04
HCO_3^{-}	ml equivalent/l	1.7	0.5
CO_3^{-}	ml equivalent/l	-	-
N	mg/Kg soil	34.2	24.8
P	mg/Kg soil	17	11
K	mg/Kg soil	188	106
Fe	mg/Kg soil	15.4	8.0
Cu	mg/Kg soil	4.06	0.86
Zn	mg/Kg soil	1.21	1.09
Mn	mg/Kg soil	1.93	0.98

*Note that clay soil is containing greater element of cation and anion compared to sandy soil.

Discussion

The phytopathogenic *P. atrosepticum* is considered the primary pathogen responsible for the rotting and wilting of stems on growing potato plants, causing soft rot and blackleg disease (Pérombelon, 2002). Recent surveys were performed to verify the source of blackleg disease in the Egyptian fields (Ashmawy *et al*., 2015). In this context, *P. atrosepticum* was isolated with other numerous species and subspecies from diseased potato plants and tubers. Few studies have been conducted to identify the phages that infect *Pectobacterium* in Egypt. Consequently, phages are considered useful environmentally friendly approaches for disease control, particularly as biological agents (Jones *et al.*, 2007). Lytic bacteriophages (phages)

are bacterial viruses that invade bacterial cells and disrupt bacterial metabolism, causing the bacterium to lyse (Sulakvelidze *et al.*, 2001). Phages are quite specific to their target bacteria, so they do not have negative effects on useful bacteria or animals and humans (Jones *et al.*, 2007; Lim *et al.*, 2015). Besides, they are self-replicating and easily prepared, enabling inexpensive production (Greer, 2005; Jones *et al.*, 2007). This study was conducted to assess the presence of lytic bacteriophage/s that have the activity against the pathogenic *P. atrosepticum* bacterium in different soil types as well as in symptomatic potato tissues (rotten tissues). All strains of *P. atrosepticum* used in this study was preliminary detected by conventional PCR using specific primers. The isolates showed a specific band at 690pb with ECA primers that targeted only *P. atrosepticum,* and that in accordance with DeBoer and Ward (1995) findings. In the present work, a lytic bacteriophage was successfully recovered and purified from only sandy soil, sampled from El-Behira governorate from a potato field in winter growing season, in which the symptoms of soft rot disease were easily observed. The frequencies of isolation of phages were low for both diseased plants and soils, particularly from diseased plants and this is in accordance with those reported by Gross *et al.* (1991) and Czajkowski *et al.* (2015). The phage could not be isolated from any of the samples of symptomatic potato tuber based on both plaque assay and spot test. No valid information is available in the literature on the success of bacteriophages recovery from (rotten) potato tubers against Pectobacterium. The aggressiveness of the phage named ϕPaP2 was confirmed as it was able to lyse the *P. atrosepticum* causing clear plaque ranged from 2.0 to 3.0 mm in diameter. Spot test also gave clear zones around the spotted phage that verifies the plaque assay results showed in Fig. (3). A previous study by Kalischuk *et al*. (2015) reported that they have isolated a lytic Peat1 phage against *P. atrosepticum.* Although, soft rotting of potato tubers is a good place to isolate phages of *P. atrosepticum*, because it can harbor high phage levels to the host. However, the results of the current study revealed that this is not always the case. Electron microscopy showed that ϕPaP2 phage belongs to the order *Caudovirales* and family *Podoviridae* based on its morphology and presence of the non-enveloped icosahedral head (diameter: 55-60 nm) and non-contractile short tail (length: 15- 20 nm) (Fig. 4). A lytic soilborne RsPod1EGY phage related to the same family (*Podoviridae*) was previously isolated against phytopathogen *Ralstonia solanacearum* by Elhalag *et al.* (2018). The same results were obtained by Smolarska *et al.* (2018) that have isolated different Podoviridae phages against *P. parmentieri.* Lytic phages have been also used to control bacterial blight of geraniums (Flaherty *et al*., 2000) and bacterial spots of tomatoes (Balogh *et al*., 2005) caused by *Xanthomonas hortorum* pv. *pelargonii*. In greenhouse and field studies, bacteriophages decreased the severity of bacterial spots of tomato to levels equal or lower than those obtained by the use of copper bactericides (Flaherty *et al*., 2000). Furthermore, the structural protein of the phage was detected by SDS-PAGE and the molecular weight of the entire head protein of the ϕPaP2 was approximately

37 kDa. The optimal MOI value of phage ϕPaP2 was determined in this study based on the average count of plaque-forming units (PFU). MOI of the phage was1.0. The average count of PFU for the optimal MOI (1.0) was higher than detected in other tested MOI. Moreover, the lytic activity of the phage against *P. atrsosepticum* was studied *in vitro*. The bacterial count (calculated based on the OD 600) showed a remarkable decrease in all tested MOI of the phage compared to pathogen control (phage- untreated) (Fig. 6). The optimal MOI of ϕPaP2 phage showed a decrease of OD of *P. atrosepticum* that reached 0.2 after 24h of incubation time. This would confirm the lytic potential of the phage against *P. atrosepticum* as a target host. Based on the one-step growth curve illustrated in Fig (7), the latent period of infection determined for ϕPaP2 phage was 50 min, and its complete cycle of infection was 150 min. As far it is known that the burst size of phages is considered as one of the evolutional traits, ϕPaP2phage has an average burst size of ~38 virions per bacterial cell. The short latency and large size of the burst of the phage may help in phage spread and increase lytic activity particularly in a high bacterial population (Chibani-Chennoufi *et al*., 2004). Moreover, phage stability was studied *in vitro* under different temperature and pH regimes and revealed that ϕPaP2 phage was stable at different pH and temperature regimes. It was able to persist at temperature 70°C, while it lost activity at a temperature higher than 70°C after incubation for 30 min. These results are in accordance with results obtained by Smolarska *et al.* (2018). The wide range of temperatures for phage stability may be a good point that the phage can efficiently infect *P. atrosepticum* in a variety of temperatures in which it naturally persists in potato fields. The phage was stable in neutral pH (pH 6.0-7.0), while the PFU of the phage was decreased in acidic and basic pHs after incubation (Fig. 9).

The specificity and host range of phage was investigated in this study against a range of different bacterial isolates. The phage was proven to be only specific to *P. atrsosepticum* as a host. The specificity of ϕPaP2 phage targeting only host bacteria gives the advantage in field application as a biocontrol agent. Moreover, it would never deleteriously affect the useful bacteria or animals and humans present in the environment (Lim *et al.*, 2015). Furthermore, the protective effect of bacteriophages on potato maceration caused by *P. atrosepticum,* was studied in potato tubers cv. Spunta. The phage ϕPaP2 was able to inhibit the growth of pathogen and to 100% decrease in potato tuber maceration caused by *P. atrosepticum* compared to the control potato tuber, inoculated with bacteria that showed soft rot after 48 h after incubation (Fig. 10). No symptoms of soft rot were detected on tubers pre-treated with the phage that was observed for 7 days after the application of bacteria and phage. These results suggest that the phage had specific great potential to be used as a bioagent against the bacterial pathogen of potato tuber during storage and transportation. No viable bacterial cells could be detected after plating on logan's medium in phage-treated tubers. Similar protection was obtained in previous studies when lytic bacteriophages were tested against *D. solani* (Czajkowski *et al*., 2014).

Similar results were reported by Lim *et al*. (2013) that a phage infecting *Erwinia. carotovora* subsp. *carotovora* could protect lettuce from soft rot disease. The use of a phage infecting *R. solanacearum* was also reported to control bacterial wilt disease (Addy *et al*., 2012). More studies are needed to better understand the potential effect of ϕPaP2 in controlling such pathogen (s) under open field conditions. Finally, the duration of activity for ϕPaP2 phage was investigated in clay and sandy soil. The long-term survival for the phage was higher in clay soil than sandy soil. The presence of pathogenic target bacterium in both clay and sandy soil showed a remarkable jump duration in the longevity of the phage compared to control soils. In this context, phages were reported in an earlier study that they have degraded rapidly in absence of host bacteria (Greer, 2005 and Jones *et al.*, 2007). The phage was able to survive shortly for 14 days in non-inoculated sandy soil compared to 22 days of survival in inoculated sandy ones. While it was survived for 20 days in non-inoculated clay soil compared to 28 days of survival in the presence of host bacterium. Bacteriophages MS2 and PRD were reported to survive for a longer period in clay than sandy soils (Straub *et al*., 1992). The chemical composition of clay soil supported the extended survival of phage in clay soil than sandy soil. Many factors were reported to function in this regard as organic matter, soil texture, cations, adsorption property, pH, ionic strength, iron oxide level, and permeability that can affect the survival of phages in soil (Keswick and Gerba, 1980). Thus, clay minerals were reported to increase the persistence of phages in soils for longer periods in the absence of their target hosts (Stotzky, 1986). The high content of Ca2+, Mg2+and Na+ detected in clay soil assumed to increase the ionic strength and thus increase the phage persistence as well as the increase in adsorption of phage on the surface of host cells (Moebus, 1996). Ionic strength is considered an important environmental factor coupling with clay particles for the survival of viruses. The aggressiveness of the phage PK-101 against *Pseudomonas solanacearum* K-101 (*Ralstonia solanacearum*) was reported to be increased with the addition of CaCl2 and MgCl2 (0.15 mol/L) (Toyoda *et al*., 1991). Formulation of isolated phage (ϕPaP2) to increase its longevity of survival and thus increasing long activity against *P. atrosepticum* will be included in further study.

In conclusion, the results presented in this study indicated that ϕPaP2 phage, isolated from Egyptian soil has the potential to be used as in biological control applications. Its lytic activity, stability, and specificity were assessed in this study. Further studies based on the estimation of genomic size and application of ϕPaP2 phage in the field are required to precisely evaluate the biological control demand on *P. atrsosepticum* affecting potato growth in fields and/ or in stores.

Conclusion

In conclusion, the results presented in this study indicated that ϕPaP2 phage, isolated from Egyptian soil has the potential to be used as in biological control applications. Its lytic activity, stability and specificity was assessed in this study.

Further studies based on the estimation of genomic size and application of ϕPaP2 phage in field to precisely evaluate the biological control demand on *P.* atrosepticum affecting potato growth in fields and/ or in stores.

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References

Addy, H.S.; Askora, A.; Kawasaki, T.; Fujie, M. and Yamada, T. 2012. Utilization of filamentous phage FRSM3 to control bacterial wilt caused by *Ralstonia solanacearum*. *Plant Dis*, **96:** 1204-1209.

Armon, R. and Kott, Y. 1993. A simple, rapid and sensitive presence/absence detection test for bacteriophage in drinking water. *J. Appl. Bacteriol.*, **74:** 490-496.

Ashmawy, N.A; Nagia, M.J; Alia, A.S and ElBebany, A.F. 2015. Identification and genetic characterization of *Pectobacterium* spp. and related *Enterobacteriaceae* causing potato soft rot diseases in Egypt. *J. Pure. Appl. Microbio.*, **9:** 1847-1858.

Balogh, B.; Jones, J.B.; Momol, M.T., and Olson S.M. 2005. Persistence of bacteriophages as biocontrol agents in the tomato canopy, p. 299-302. In Momol, M.T.; Ji, P., and Jones, J.B. (ed.), Proc. 1st Int. Symp. Tomato Dis. International Society for Horticultural Science. *Acta Hortic*. 695.

Carlson, K. and Miller, E. 1994. Experiments in T4 genetics. In: Karam, J.D., editor. Bacteriophage T4. ASM Press, Washington DC, USA, p. 419-483.

Czajkowski, R.; Ozymko, Z.; de Jager, V.; Siwinska, J.; Smolarska, A.; Ossowicki, A.; Narajczyk M. and Lojkowska, E. 2015. Genomic, proteomic and morphological characterization of two novel broad host lytic bacteriophages fPD10.3 and fPD23.1 infecting pectinolytic *Pectobacterium* spp. and *Dickeya* spp. *PLOS ONE,* **10**: e0119812.

Czajkowski, R.; Ozymko, Z. and Lojkowska, E. 2014. Isolation and characterization of novel soilborne lytic bacteriophages infecting Dickeya spp. biovar 3 (‘D. Solani’). *Plant Pathol.*, **63:**758-772.

Chibani-Chennoufi, S.; Bruttin, A.; Dillmann, M.L. and Brüssow, H. 2004. Phage-host interaction: An ecological perspective. *J. of Bacteriol.*, **186:** 3677-3686.

De Boer, S.H and Ward L.J. 1995. PCR detection of *Erwinia carotovora* subsp. *atroseptica* associated with potato tissue. *Phytopathol*, **85(8):** 854-858.

Elhalag, K.; Nasr-Eldin, M.; Hussien, A. and Ahmad, A. 2018. Potential use of soilborne lytic Podoviridae phage as a biocontrol agent against *Ralstonia solanacearum*. *J. Basic Microbiol.*, **58:**658-669.

Elhalag, K.; Emara, H.; Messiha, N.A.S. and Elhadad, S.A. 2015. The relation of different crop roots exudates to the survival and suppressive effect of *Stenotrophomonas maltophilia* (PD4560), biocontrol agent of bacterial wilt of potato. *J Phytopathol*, **163:** 829-840.

Fahy, P.C. and Hayward, A.C. 1983. Plant Bacterial Disease (a Diagnostic Guide). Academic Press, Sydney, New York, London. PP 349.

Farag, S.; Elhalag, K.M.; Hagag, M.H.; Khairy, A.M.; Ibrahim, H.M.; Saker, M.T. and Messiha, N.A.S. 2017. Potato bacterial wilt suppression and plant health improvement after application of different antioxidants. *J. Phytopathol.*, **165:** 522-537.

Flaherty, J.E.; Jones, J.B.; Harbaugh, B.K.; Somodi, G.C. and Jackson, L.E. 2000. Control of bacterial spot on tomato in the greenhouse and field with h-mutant bacteriophages. *Hort Science*, **35:** 882-884.

Gasic, K.; Ivanovic, M.M.; Ignjatov, M.; Calic, A. and Obradovic, A. 2011 Isolation and characterization of *Xanthomonas euvesicatoria* bacteriophages. *J. Plant. Pathol.*, **93:** 415-423.

Greer, G.G. 2005. Bacteriophage control of foodborne bacteria. *J. Food Protec.*, **68:** 1102-1111.

Gross, D.C.; Powelson, M.L.; Regner, K.M. and Radamaker, G.K. 1991. A bacteriophage-typing system for surveying the diversity and distribution of strains of *Erwinia carotovora* in potato fields. *Phytopathol.,* **81:** 220-226.

Hayward, A.C. 2000. *Ralstonia solanacearum*, In: Lederberg, J. (ed.). Encyclopedia of Microbiology; 4: p.32-42. Academic Press, San Diego.

Jamal, M.; Hussain, T.; Rajanna Das, C. and Andleeb, S. 2015. Isolation and characterization of a *Myoviridae* MJ1 bacteriophage against multi-Drug resistant *Escherichia coli* 3. *Jundishapur. J. Microbiol.*, **8:** 1-8.

Jee, S.; Malhotra, S.; Roh, E.; Jung, K.; Lee, D.; Choi, J.; Yoon, J. and Heu, S. 2012. Isolation of bacteriophages which can infect *Pectobacterium carotovorum* subsp. *carotovorum*. *Res. in Plant. Dis.*, **18:** 225-230.

Jones, J.B.; Jackson, L.E.; Balogh, B.; Obradovic, A.; Iriarte, F.B. and Momol, M.T. 2007. Bacteriophages for plant disease control. *Ann. Rev. Phytopathol.*, **45:** 245-262.

Kalischuk, M.; Hachey, J. and Kawchuk, L. M. 2015. Complete genome sequence of phytopathogenic *Pectobacterium atrosepticum* bacteriophage Peat1. *Genome Announcements*, **13**, e00760–e00715.

King, E.; Ward, M. and Raney, D. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. *J. Lab Clin. Med*., **44**: 301-307.

Keswick, B.H and Gerba, C.P. 1980. Viruses in groundwater. *Environ. Sci. Technol.*, **14:** 1290-1297.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nat.*, **227:** 680-685.

Lim, J.A.; Lee, D.H. and Heu, S. 2015. Isolation and genomic characterization of the T4-like bacteriophage PM2 infecting *Pectobacterium carotovorum* subsp. *carotovorum*. *The Plant Pathol. J.*, **31:** 83-89.

Lim, J.A.; Jee, S.; Lee, D.H.; Roh, E.; Jung, K.; Oh, C. and Heu, S. 2013. Biocontrol of *Pectobacterium carotovorum subsp. carotovorum* using bacteriophage PP1. *J. Microbiol. Biotechnol.*, **23:** 1147-1153.

Mihara, T.; Nasr-Eldin, M.A; Chatchawankanphanich, O. and Bhunchoth, A. 2016. A *Ralstonia solanacearum* phage ϕRP15 is closely related to Viunalike viruses and encodes 19 tRNA-related sequences. *Virol. Rep.*, **6:** 61-73.

Moebus, K. 1996. Marine bacteriophage reproduction under nutrient-limited growth of host bacteria. II. Investigations with phage–host system [H3:H3/1]. *Mar. Ecol. Prog. Ser.,* **144:** 13-22.

Pérombelon, M.C. 2002. Potato diseases caused by soft rot erwinias: an overview of pathogenesis. *Plant Pathol.*, **51:** 1-12.

Sambrook, J.; Fritsch, E.F and Maniatis, T. 1989. Molecular cloning: a laboratory manual, 2nd ed Cold Spring Harbor Laboratory, Cold Spring Harbor NY. 545 pp.

Smolarska, A.; Rabalski, L.; Narajczyk, M. and Czajkowski, R. 2018. Isolation and phenotypic and morphological characterization of the first Podoviridae lytic bacteriophages ϕA38 and ϕA41 infecting *Pectobacterium parmentieri* (former *Pectobacterium wasabiae*). *Eur. J. Plant Pathol.*, **150:** 413-425.

Stotzky, G. 1986. Influence of soil mineral colloids on metabolic processes, growth, adhesion, and ecology of microbes andviruses. In: Interactions of Soil Minerals with Natural Organics and Microbes. Eds. Huang, P.M. and Schnitzer, M, pp. 305-428. Soil Science Society of America, Madison.

Straub, T.M.; Pepper, I.L. and Gerba, C.P. 1992. Persistence of viruses in desert soils amended with anaerobically digested sewage sludge. *Appl. Environ. Microbiol.*, **58:** 636-641.

Sulakvelidze, A.; Alavidze, Z. and Glenn Morris Jr, J. 2001. Bacteriophage Therapy. *Antimicrobial Agents Chemotherapy*. **45:** 649- 659.

Toyoda, H.; Kakutani, K.; Ikeda, S.; Goto, S., Tanaka, H. and Ouchi, S. 1991. Characterization of deoxyribonucleic acid of virulent bacteriophage and its infectivity to host bacteria, *Pseudomonas solanacearum*. *J. Phytopathol.*, **131:** 11-21.

Van der Wolf, J.M. and De Boer, S.H. 2007. Bacterial pathogens of potato. In: Vreugdenhil, D.; Bradshaw, J.; Gebhardt, C.; Govers, F.; MacKerron, D.K.L.; Taylor, M.A. and Ross, H.A. (eds). Potato Biology and Biotechnology, pp. 595-617. Elsevier academic press, San Diego, USA.

Yamada, T.; Kawasaki, T.; Nagata, S.; Fujiwara, A. 2007. New bacteriophages that infect the phytopathogen *Ralstonia solanacearum*. *Microb.*, **153:** 2630-2639.

Corresponding author: Elhalag, K.M.
E-mail: kamel_moon_82@yahoo.com

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عزل وتعريف بكتريوفاج جديد لمقاومة بكتريا *Pectobacterium atrosepticum*

كامل محمد الحلاج

قسم بحوث الأمراض البكتيرية ، معهد بحوث أمراض النباتات ، مركز البحوث الزراعية ، الجيزة ، مصر

تم عمل محاولات عديده فى هذ الدراسه من اجل عزل وتنقية بكتريوفاج له خصائص محلله لبكتريا *Pectobacterium atrosepticum* والمعروف سابقا باسم *Erwinia carotovora sub sp atroseptica*. حيث ان هذا المسبب المرضى يعتبر من الممرضات النباتيه الشائعه والتى تسبب خسائر اقتصاديه فى مصر والعالم. من اجل تطوير عامل حيوى فعال ضد المسبب المرضى للعفن الطرى والساق السوداء فى النباتات من العائله الباذنجانيه, تم عزل البكتريوفاج (ϕPaP2) والذى ينتمى الى العائله *Podoviridae* من التربه المصريه فى دلتا النيل. واشتملت الدراسه معرفة الخصائص المورفولوجيه و الوبائيه وكذلك النشاط المحلل لهذا الفاج. حيث تم عزل وتنقية هذا الفاج باتباع طرق single plaque isolate approach وبعد ذلك تم اكثاره فى معلق بكتيرى تم حقنه بانسب تركيز من الفاج للتضاعف داخل الخليه البكتيريه Optimal Multiplicity of Infection (MOI). اعطى الفاج حجم من plaque يتراوح قطرها من ٢-٣ مم. أوضحت نتائج الفحص الظاهرى باستخدام الميكروسكوب الالكترونى ان الفاج يتكون من راس يتراوح قطرها من ٥٥-٦٠ نانوميتر وذيل قصير يتراوح طوله من ١٥-٢٠ نانوميتر والذى قد ينتمى الى العائله *Podoviridae order caudovirales*. تم تحديد الوزن الجزيئى للتركيب البروتينى لراس الفاج باستخدام الفصل الكهربائى SDS–PAGEحيث كان ٣٧ كيلو دالتون تقريبا. سوف يتم دراسة الحجم الجينومى والوظائف الجينيه للفاج فى دراسات مستقبليه.

أوضحت النتائج ان انسب تركيز من الفاج للتضاعف داخل الخليه البكتيريه هو ١,٠ بناءا على متوسط اعداد plaque forming units (PFU). أوضحت نتائج منحنى النمو للفاج ان فترة العدوى الخفيه للفاج latent period of infection كانت حوالى ٥٠ دقيقه بنما كانت دورة العدوى الكامله حوالى ١٥٠ دقيقه وكان حجم الانفجار داخل الخليه البكتيريه حوالى ٣٧ فاج. أوضحت النتائج المعمليه ان الفاج يحتفظ بنشاطه وثباته عند درجة ٧٠ درجه مئويه بينما يفقد الفاج نشاطه عندما تزيد درجة الحراره عن ٧٥ درجه مئويه. أوضحت النتائج ايضا ان انسب اس هيدروجينى للفاج هو ٧ بينما كان نشاط الفاج مابين ٤-٧ اس هيدروجينى. تم اختبار المدى العوائلى للفاج على حوالى ١٨عزله بكتيريه منهم ٧ عزلات ينتمو الى *P. atrosepticum* وايضا عدد ١١ عزله بكتيريه اخرى لها خصائص ممرضه للبطاطس وايضا عزلات ذات خصائص

تضاديه تم عزلها سابقا. تم دراسة النشاط المحلل للفاج فى النبات حيث ادى الفاج الى تقليل اعراض العفن الطرى على درنات البطاطس والمسبب لها بكتيريا *P. atrosepticum* بالمقارنه الى الدرنات التى لم تعامل بالفاج والتى تم معاملتها فقط بالبكتريا الممرضه. تم دراسة مدة بقاء الفاج فى انواع تربه مختلفه (تربه طينيه واخرى رمليه). فى العموم كانت مدة بقاء الفاج فى التربه الطينيه اعلى من التربه الرمليه. كان لوجود العائل البكتيرى فى التربه تأثير ملحوظ على مدة بقاء الفاج فى التربه بالمقارنه بالتربه التى لم تعامل بالبكتريا. ونستنتج من نتائج الدراسه ان الفاج المعزول كان له القدره على ايقاف نمو البكتريا الممرضه فى الاختبارات المعمليه وكذلك حماية درنات البطاطس من التحلل بفعل البكتريا الممرضه.