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RESEARCH PAPER

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Potential use of soilborne lytic Podoviridae phage as a biocontrol agent against *Ralstonia solanacearum*

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A new podovirus RsPod1EGY Ralstonia phage (GenBank accession no MG711516) with a specific action against *R. solanacearum* phylotype IIa, sequevar I (race 3, biovar 2) was isolated from Egyptian soil. The potential efficacy of the isolated phage to be used as biocontrol agent was evaluated *in vitro* and under greenhouse conditions. The podovirus phage produced a plaque size of 3.0–4.0 mm in diameter and completed its infection cycle in 180 min after infection with a burst size of ~27 virions per infected cell. On the basis of restriction endonuclease analysis, the genome size of the phage was about 41 kb of double-stranded DNA. *In vitro* studies showed that RsPod1EGY is stable at higher temperatures (up to 60 °C), and at a wide pH range (5–9). SDS–PAGE analysis indicated the major structural protein to be approximately 32 kDa. Bacteriolytic activity of RsPod1EGY against *R. solanacearum* was detected at different multiplicity of infection (MOI). RsPod1EGY proved to be effective in reduction and prevention of formation of surface polysaccharides of *R. solanacearum*, during the exponential growth phase of the latter. Interestingly, RsPod1EGY was effective in suppression of *R. solanacearum* under greenhouse conditions. All Phage-treated tomato plants showed no wilt symptoms or any latent infection during the experimental period, whereas all untreated plants have wilted by 10 days post-infection. The lytic stability of RsPod1EGY phage at higher temperature as well as its effective suppression of wilting symptoms under greenhouse conditions would contribute to biocontrol the bacterial wilt disease in Egypt under field conditions.

KEYWORDS
biological control, disease severity, lytic bacteriophage, podoviridae, potato brown rot

1 | INTRODUCTION

Bacterial wilt disease caused by the gram-negative β-proteobacterium; *Ralstonia solanacearum*; caused serious losses in many important crops including tomato, potato, tobacco, and eggplant. In Egypt, potato (*Solanum tuberosum*) is an important staple crop and represents the second most important crop for exportation, and is the main crop affected by this disease, especially in Nile Delta regions. The causal agent is a quarantine pathogen with zero tolerance in import

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and export, and its presence is hindering the smooth flow of the Egyptian potatoes to different international markets, especially to EU countries and Russia [1]. In the past, *R. solanacearum* was subdivided into five races based on host range and into five biovars based on physiological and biochemical characteristics [2]. Recently, four phylotypes based on phylogenetic analysis of nucleoid sequences of particular genes were used for classification and were related to the geographic origin [3]. The only reported causal agent of bacterial wilt in the Nile Delta of Egypt is *R. solanacearum* Phylotype IIa, sequevar 1, race 3, biovar 2 [4]. The more recent outbreaks of Phylotype II, sequevar 1adapt in several EU countries as well as establishment in Egypt seems to be related to latent infections carried on imported potatoes from regions where the disease is endemic [5]. Pathogenicity determinants of *R. solanacearum* include diverse genes responsible for colonization and wilting of host plants, such as those coding for exopolysaccharide (EPS), lytic enzymes, effector proteins, and type III secretion system (T3SS), hypersensitive reaction and pathogenicity (*hrp*) and others [6]. There is an intense relationship between *R. solanacearum* LPS and EPS, both of them are important for *R. solanacearum* pathogenicity especially in the early stages of infection and also function in adhesion to host surfaces. One of the most important of these products is an acidic, high molecular mass extracellular polysaccharide (EPS). EPS was suggested that is the cause of wilting in infected plants, as it blocks the vascular system and thereby interfere with water movement [7].

There is an urgent need to develop a novel method to manage the bacterial wilt of potato in Egypt due to the economic importance of potato crop in Egypt. Exportation of Egyptian potatoes to the EU is only allowed from specially designated and tested uncontaminated areas known as Pest-Free Areas (PFAs). There are no effective chemicals available to eradicate the bacterial pathogens from soil or water, nor to cure infected plants. Cultivation of resistant cultivars could be a solution, although there are no resistant cultivars available [8]. The use of both *Stenotrophomonas maltophilia* and salicylic acid as biotic and abiotic elicitors for improving plant immune response proved to have a suppressive effect on the dominant race of *R. solanacearum* in Egypt under greenhouse conditions [9].

Bacterial viruses, bacteriophages, have proved to be efficient biological nanomachines designed to infect their hosts with high efficacy and specificity. Various phages have been assessed as biological control against different plant diseases [10], and some have also been isolated and evaluated as biocontrol agents against bacterial wilt caused by *R. solanacearum* phylotype 1 (race 1) isolated from tobacco in Japan [11–13]. Yamada et al. [11], Fujiwara et al. [12], and Askora et al. [13] isolated and characterized various kinds of phage that can infect and interact with different races of *R. solanacearum*, whereas ϕRSY1 phage could infect strains from races 1,3, and 4 in biovars 3, 4, and N2, respectively while ΦRSA1 had a narrower specificity. Phages infecting *R. solanacearum* were proved to be possible control agents against wilt disease [12]. Moreover, the main advantage of using a phage as a biocontrol agent is the possible reduction in the usage of ineffective chemical pesticides. Phages show a narrow specificity of action by killing only the targeted bacterial pathogenic species, and thus avoid the side effect of elimination of non-target microorganisms that could represent important components of the agroecosystem. In this study, different soil samples from different localities in the Nile Delta of Egypt were collected and used for isolation of phage active against the only dominant race of *R. solanacearum* in Egypt Phylotype IIa, sequevar 1. The potential efficacy of the isolated phage as a biocontrol agent was evaluated *in vitro* and under greenhouse conditions. The *in vitro* evaluation was based on studying the conditions of optimal multiplicity of infection (MOI) that favor the lytic activity of the isolated phage against *R. solanacearum* and also the estimation of production of surface polysaccharides. Its physical properties and protein composition were also investigated in this study. Molecular techniques including RFLP were used for characterizing and determining the approximate genome size of the phage. Its host range was evaluated against some strains of *R. solanacearum* belonging to phylotype 11 and also against some saprophytic and pathogenic bacteria belonging to different genera isolated from soil. Finally, a greenhouse evaluation of the activity of the phage was based on determining the disease severity of tomato plants after treatment with isolated phage comparing to the pathogen control treatment as well as detecting the pathogen in different plants roots using real time PCR and plating on semi selective medium.

2 | MATERIALS AND METHODS

2.1 | Bacterial cultures and growth conditions

Eight highly virulent strains of *R. solanacearum* Phylotype IIa, sequevar 1 (race 3, biovar 2); K3, K9, K10, K11, K12, K16, K17, and K19 were used in this study and were originally isolated in Egypt from symptomatic potato tubers, showing typical brown rot symptoms (brownish discoloration of vascular bundles with slimy whitish bacterial ooze) and maintained at the culture collection of Potato Brown Rot Project (PBRP), Ministry of Agriculture, Giza, Egypt; previously identified by ref. [14,15]. Strain K3 was used for isolation of the phage, while other strains were used for evaluation of the host range and the activity of the isolated phage. The other bacterial strains listed in Table 1 were kindly donated by Science and technology development Funding (STDF) project no. 2905. These strains include plant pathogenic bacteria and antagonistic bacteria isolated from

TABLE 1 Host range of RsPod1EGY phage using a range of bacterial species and strains

Code	Bacterial strains[a]	Spot test	Plaque assay
K3	*Ralstonia solanacearum* Phylotype IIa sequevar I	+	+
K9,	*Ralstonia solanacearum* Phylotype IIa sequevar I	+	+
K10	*Ralstonia solanacearum* Phylotype IIa sequevar I	+	+
K11	*Ralstonia solanacearum* Phylotype IIa sequevar I	+	+
K12	*Ralstonia solanacearum* Phylotype IIa sequevar I	+	+
K16	*Ralstonia solanacearum* Phylotype IIa sequevar I	+	+
K17	*Ralstonia solanacearum* Phylotype IIa sequevar I	+	+
K19	*Ralstonia solanacearum* Phylotype IIa sequevar I	+	+
1	*Serratia marcescens*	–	–
2	*Pseudomonas fluorescens*	–	–
3	*Stenotrophomonas maltophilia*	–	–
4	*Bacillus thuringiensis*	–	–
100	*Pectobacterium carotovorum*	–	–
200	*Pectobacterium atrosepticum*	–	–
300	*Dickeya dianthicola*	–	–
400	*Dickeya solani*	–	–
177	*Pseudomonas aeruginosa*	–	–
114	*Enterobacter aerogenes*	–	–
447	*Pseudomonas japonica*	–	–

Sensitivity to RsPod1EGY (+) sensitive, (–) resistant.

[a]All *R. solanacearum* strains used were previously isolated and identified by ref. [13,14] and other bacterial strains used in this study were kindly provided by STDF project No. 2905 in Egypt.

soil against *R. solanacearum* Phylotype IIa, sequevar 1. Bacterial strains were continuously cultured on nutrient agar slants for short-term subculturing (two subculturing), while long preservation was done at −30°C using sterilized glycerol buffer (20%) [2].

2.2 | Soil samples and plaque assay

Soil samples used for bacteriophage isolation were collected from cropping fields in Qalubyia, Behira, and Monufyia Governorates in the Nile Delta of Egypt. Sampling was performed in the summer season. Samples of 5 g soil were suspended in 50 ml of CPG medium containing 0.1% casamino acids, 1% peptone, and 0.5% glucose [16] previously inoculated with *R. solanacearum* strain K3 with a 5×10^8 CFU/ml. The inoculated flasks were incubated for 12–16 h in a shaker incubator (SHKE481HP, Thermo-Scientific, USA) at 125 rpm at 28 °C. The resulting cell suspension was centrifuged for 10 min at 3000 rpm. The supernatant was then filtered through a membrane filter (0.45 µm pore size; Steradisc, Kurabo Co. Ltd., Osaka, Japan). Aliquots of 100 µl of the suspension were used for a plaque forming assay, using strain K3 (Table 1) of *R. solanacearum* Phylotype II, sequevar 1 (race 3 biovar 2), as the host. The plaque assay was carried out according to [17] using CPG plates containing 2% agar and overlaid with 0.75% CPG soft agar. The plates were incubated at 28 °C for 24 or 48 h. The shape, size and number of plaques were recorded after the incubation period.

2.3 | Purification and propagation of phage

Phage was isolated and purified using single plaque isolates, and was then propagated as follow: a culture of bacterial cells (16–24 h grown in a litre of liquid CPG medium and incubated at 28 °C with a concentration of 5×10^8 CFU/ml in concentration) was diluted 100-fold with 100 ml fresh CPG medium in a 500 ml flask. The phage from the original isolation plates was added at a multiplicity of infection (MOI) of 0.1, 0.5, and 1.0, when the cultures reached 0.2 units at OD_{600}. After 20 h of incubation, the cells were removed by centrifugation using a refrigerated centrifuge (SIGMA 3-18 K, Sigma Laborzentrifugen GmbH, Germany) at 8800×*g* (6000 rpm) for 15 min at 4 °C. The supernatant was passed through a 0.2 µm membrane filter (Gelman Sciences, USA) and phage particles were precipitated by centrifugation at 20,400×g (13,500 rpm) for 1 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 generally 2.5×10^{11} PFU/ml that was determined using plaque assay. Purified phages were stored at 4 °C for further study.

2.4 | Optimal multiplicity of infection

The optimal ratio of infection between the propagated phage particles and host bacterial cells (*R. solanacearum* strain K3) was determined according to methods described by ref. [18]. The *R. solanacearum* culture was infected with the phage at different (MOI) (0.1, 0.5, and 1). The optimal MOI that resulted in the highest phage titre (the highest PFU/ml) was then determined. The experiment was repeated three times and the average of results from all repetitions were estimated.

2.5 | Determination of phage host range

The host range of the isolated phage was evaluated against a restricted panel of other seven *R. solanacearum* strains, belongs to Phylotype IIa, sequevar 1, and also against some other plant pathogenic bacteria as well as antagonistic bacterial strains isolated from soil by ref. [19], namely *Serratia marcescens*, *Pseudomonas fluorescense, Stenotrophomonas maltophilia, Bacillus thuringiensis, Pectobacterium carotovorum, Pectobacterium atrosepticum, Dickeya dianthicola, Dickeya solani, Pseudomonas aeruginosa, Enterobacter aerogenes, Pseudomonas japonica*, (kindly provided by STDF project No. 2905 in Egypt, listed in Table 1). The Phage host ranges were examined using both the spot test [20] and plaque assay [17]. Briefly, for the spot test, to three ml of soft agar (0.75%) 200 µl of a 24 h culture bacteria grown at 28 °C (cell density of 10^8 CFU/ml) was added, gently mixed and poured onto the surface of CPG medium. A single drop (5 µl) of phage suspension (2.5×10^{11} PFU/ml) was spotted on the inoculated CPG plates, which were incubated overnight at 28 °C. Lysed areas on the spot inoculations were observed until 48 h to indicate observe lysis efficacy. The plaque assay was done using 100 µl of phage suspension (with the same concentration mentioned above) added to 250 µl of tested bacterial strains listed in Table 1 (OD_{600} 0.2) in a tube containing 3 ml of CPG- soft agar. The mixture was poured onto the surface of CPG agar plates containing 2% agar and allowed to solidify for 30 min. The plates were subsequently incubated for 24 h at 28 °C and plaque formation were determined. Non-phage inoculated plates were used as a control. The phage was also sent to Floral and Nursery Plants Research Unit, National Arboretum, Agricultural Research Service, USA to be tested its activity against other strains belonging to phylotype 1 and phyltotype II (race 1 and race 3, respectively) and further studies.

2.6 | One step growth curve of the phage

One-step growth curve experiments were carried out according to ref. [21,22], with some modifications. *R. solanacearum* K3 strain was used as host for the *R. solanacearum* specific phage that were isolated. Bacterial cells suspension grown on CPG broth (0.3 U of OD_{600}, 24 h incubation period at 28 °C) were concentrated by centrifugation and resuspended in 10 ml of fresh CPG (ca. 1×10^8 CFU/ml). Phage was added with its optimal MOI (0.5) and allowed to adsorb for 30 min at 28 °C. After the phage adsorption period, the mixture was centrifuged and the pellet was resuspended in CPG. The bacterial cells were incubated at 28 °C. Every 30 min following incubation, samples were taken at intervals up to 5 h and immediately diluted for titer determination using the double-layered agar plate method according to [17] the whole experiment was repeated twice. The resulting growth curve was expressed as the ratio of PFU per infected cell during each time interval.

2.7 | Extraction of phage DNA

Phage DNA was extracted from the purified phage particles by standard molecular biological techniques for phage-DNA isolation using the phenol extraction method according to [23]. Purity and concentration of DNA were determined using a spectrophotometer (NanoDrop 1000 spectrophotometer, Thermo Fisher Scientific, Wilmington, USA) according to ref. [24].

2.8 | Restriction endonuclease analysis of phage DNA

One µg of purified phage DNA was subjected for digestion with seven different restriction enzymes (*Bam*H1, *Eco*RI, *Cla*I, *Not*I, *Acc*I, *Spe*I, and *Hind*III) according the manufacturer's instructions (BioLabs, USA) and analyzed using 0.8% agarose gel. Restriction analysis using *Bam*H1 was studied in a separate gel. The size of phage genome was estimated by compilation of DNA fragment sizes resulting from restriction enzymes digestion profiles [25]. Isolated phage was sent to the Floral and Nursery Plants Research Unit, National Arboretum, Agricultural Research Service, USA and whole genome sequencing was performed in separate study.

2.9 | *In vitro* stability of phage at different temperatures and pH

The phage stability at different temperature and pH conditions were performed *in vitro* according to methods described by [26] with some modifications. The phage titre was first determined as described above. Then, 1.5 ml Eppendorf tubes

containing diluted suspension of the bacteriophage were incubated at 28 °C, 50 °C, 60 °C, 70 °C, 80 °C and 90 °C for 30 min [27]. The plaque assay as described above was done as three replicates for each tested temperature. We assayed phage stability in CPG broth at pH values 2, 5, 7, 9, and 11, after incubation for 3 h at 28 °C, the survived phage particles were counted immediately using double-layer agar plate method as described above.

2.10 | Protein quantification

Phage protein was quantified using a spectrophotometer (NanoDrop 1000 spectrophotometer, Thermo Fisher Scientific) at ratio 260/280 nm wavelengths, to indicate the purity of phage protein.

2.11 | Analysis of phage proteins

The different structural proteins of phage were separated using 12% SDS–polyacrylamide gel electrophoresis (PAGE) (BioGmetra, Germany) according to ref. [28]. The test was performed at Cairo University Research Park (CURP), Giza, Egypt. Coomassie brilliant blue (0.5%) was used to stain the gel, then de-stained for best visibility of the protein bands with several changes of de-staining solution and stored in 7% glacial acetic acid solution for photography.

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

2.12 | *In vitro* bacteriolytic activity of phage related to the growth curve and production of surface exopolysaccharides of *R. solanacearum*

Bacteriolytic activity was tested at 0.1, 0.5 and 1 multiplicity of infection (MOI). A *R. solanacearum* suspension (50 ml) of culture in CPG medium in the exponential growth phase [optical density (OD) 600 = 0.3–10^8 cell/ml] was mixed with the phage at the different MOI. The mixtures were incubated in a shaker incubator (SHKE481HP, Thermo-Scientific) at 28 °C and 100–150 rpm. The optical density of the mixtures was measured using a spectrophotometer (Jenway 6300, UK) OD 600 at 0, 2, 4, 6, 8, 10, 12, and 24 h post-infection in three replicates for each time interval.

Surface polysaccharides were extracted and estimated from the same mixture (bacterial cells with phages) at 0, 4, 8, 12, and 24 h post-infection at different multiplicity of infection (MOI) (0.1, 0.5, and 1) according to ref. [29] as three replicates for each time interval. Non-phage treatments were used as negative control. Concentrations of *R. solanacearum* surface polysaccharides were calculated from the standard curve according to Brimacombe and Beatty [29].

2.13 | *In planta* biocontrol activity of phage against *R. solanacearum* under greenhouse conditions

A mixed suspension (1:1:1) of three virulent strains (K3, K10, K16) of *R. solanacearum* Phylotype IIa, sequevar 1; tested before for their virulence on tomato plants by a bioassay test according to [30]; was used to inoculate soil at a final concentration of 5×10^8 CFU/g dry soil, which was used to test the potential use of phage as a biocontrol agent. Four week old tomato seedlings 4 (*Solanum lycopersicum* cv. Pinto, 3–5 leaves/seedling), cultivated in pots under controlled greenhouse conditions (28–30 °C, 75–85% RH, 16 h light/8 h dark) were used for the bioassay test. Soil was infested with equal volumes of the mentioned mixture of virulent strains [9]. The ratio of inoculums/soil was 1:10 (v/w). Tomato roots were gently cut to facilitate pathogen penetration and improve the artificial inoculation. Four pots mixed with a phage suspension (5 ml, containing 2×10^{11} PFU/ml), added 1 day before bacterial challenge were used for the phage treatments. Four pots were inoculated with *R. solanacearum* strains mixture only, and were used as positive control. Four non-inoculated pots were used as healthy, negative control. The experiment was repeated three times with four replicates for each treatment.

Wilting symptoms were observed and recorded daily and graded from 1 to 5 as described by Kempe and Sequeira [31]. Disease severity was recorded daily till the positive control plants wilted completely. Area under disease progress curve (AUDPC) was also calculated. Population level of the pathogen was detected in the crown area of the tomato plants in each treatment after 10 days from soil inoculation using a serial dilution plating technique on SMSA medium as modified by Elphinstone et al. [32]. Also, molecular detection of *R. solanacearum* DNA in the extract of tomato crown area was done by real time PCR to detect any latent infection according to ref. [33] using specific primers and probe. DNA extraction was performed using a semi-automated DNA extractor (Biosprint 15, Qiagen) following the manufacture instructions. Cycle threshold (Ct) values were detected, where Ct > 40 was considered a negative result (*R. solanacearum* not detected) according to the manufacture's protocol. PCR was carried out in 25-ml volumes after DNA extraction using MicroAmp Optical Caps and MicroAmp Optical 96-well reaction plates (Applied Biosystems, USA). The program cycles were set as follows: 2 min at 50 °C, 10 min at 95 °C, followed by 40 two-step cycles of 10 s at 95 °C and finally 1 min at 60 °C.

3 | RESULTS

3.1 | Isolation and purification of the phage

A new lytic podovirus phage with activity against *R. solanacearum* phylotype IIa sequevar I was successfully isolated from Egyptian soil samples after four trials The same phage was isolated from two different soil samples originated from fields in Talia, Monufia Governorate and Sendione, Qalubyia Governorate. The most recent cropping systems practiced in the soil fields from which the phage had been isolated was potato and corn (*Zea mays*), respectively. Circular clear plaques were formed on the lawn of the host, which demonstrates the virulent activity of the lytic phage. The plaque size diameter ranged from 3.0 to 4.0 mm with intact boundaries (Fig. 1). The isolated phage was designated as phage RsPod1EGY (GenBank accession no MG711516). The morphology of RsPod1EGY was previously characterized in a separate study by an icosahedral head of 60 nm in diameter and a short tail of 15 nm in length, typical of a podovirus order caudovirales.

3.3 | The host range of RsPod1EGY

Eighteen soil-isolated bacterial strains, belonging to different gram-positive and gram-negative genera, listed in Table 1, were tested for susceptibility to RsPod1EGY infection. All these strains were insensitive to the phage except all *R. solanacearum* strains used in this experiment (K3, K9, K10, K11, K12, K16, K17, and K19) according to both the spot test and plaque assay results. These results indicate that the phage is possibly specific to *R. solanacearum* (phylotype IIa, sequevar 1, race 3, biovar 2).

3.4 | Optimal multiplicity of infection (MOI)

The optimal MOI value of phage RsPod1EGY was 0.5 based on the average count of plaque forming units (PFU) from three separate determinations. The average count of PFU for the optimal MOI (0.5) was 2.3×10^{11} PFU/ml, while in case of 1.0 and 0.1 MOI; the average count of PFU was 1.2×10^{11} and 1.7×10^{10} PFU/ml, respectively.

3.5 | One step growth curve

The obtained single-step growth curve (Fig. 2) indicated that the latent period of infection for RsPod1EGY was 60 min, while the complete cycle of infection was 180 min. The phage has an average burst size of ~27 virions per bacterial cell.

3.6 | Restriction endonuclease digestion profiles of RsPod1EGY

The genomic DNA of RsPod1EGY, digested by bamH1 resulted in two restriction bands (Supporting Information Fig. S1). Whereas when digested by *Eco*RI, *Cla*I, *Not*I, *Acc*I, *Spe*I, and *Hind*III different band patterns of different sizes were obtained (Fig. 3), indicating that the phage nucleic acid is double-stranded DNA. The sum of the restriction fragment sizes suggests that the bacteriophage genome to be approximately 41 kbp in length.

3.7 | Stability of RsPod1EGY under different temperatures and pH

The phage sensitivity to various temperatures is represented in Fig. 4a. The activity of phage RsPod1EGY remained stable in a range of temperature up to 60 °C, but was lost when RsPod1EGY was subjected to temperatures of 70, 80 and 90 °C for 30 min. Phage RsPod1EGY retained its lytic capability for up to 3 h at pH ranging from 5 to 9, when incubated at 28 °C and showed maximum stability at pH 7, while; at pH 2 and pH 11 no plaques were detected. The number of plaques was found to increase with increasing pH, reaching the highest number at pH 7 (Fig. 4b).

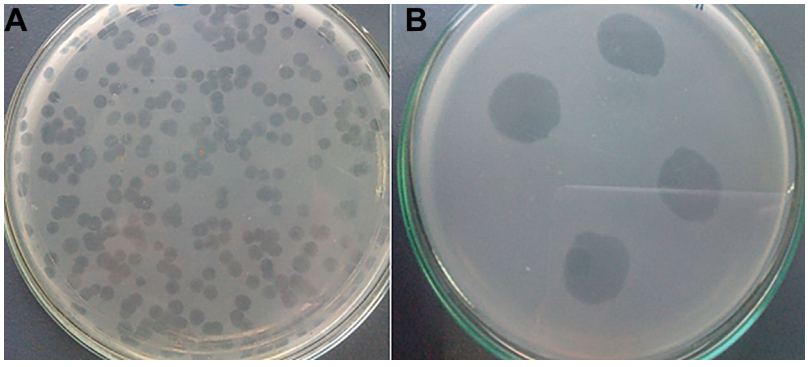

FIGURE 1 (A) A higher dilution (10^{-8}) of the RsPod1EGY phage titre showing clear plaques 3.0–4.0 mm in diameter and (B) spot test showing phage lytic activity

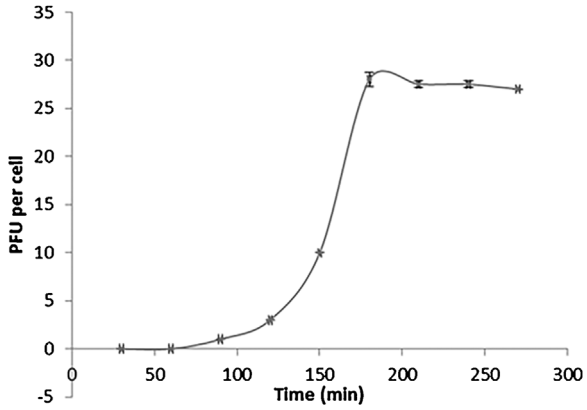

FIGURE 2 One-step growth curve for RsPod1EGY phage infecting *R. solanacearum* race 3 biovar 2. Note: Latent time and burst size of phage RsPod1EGY were 60 min and 27 PFU per cell respectively. Error bars represent ± the standard deviation

3.8 | SDS–PAGE of structural proteins of RsPod1EGY

SDS–PAGE of the phage protein, showed that RsPod1EGY phage is composed of in total of eight proteins. Phage protein molecular weights ranged approximately from 22 to 170 kDa (Fig. 5). The molecular weight of the major head protein is approximately 32 kDa.

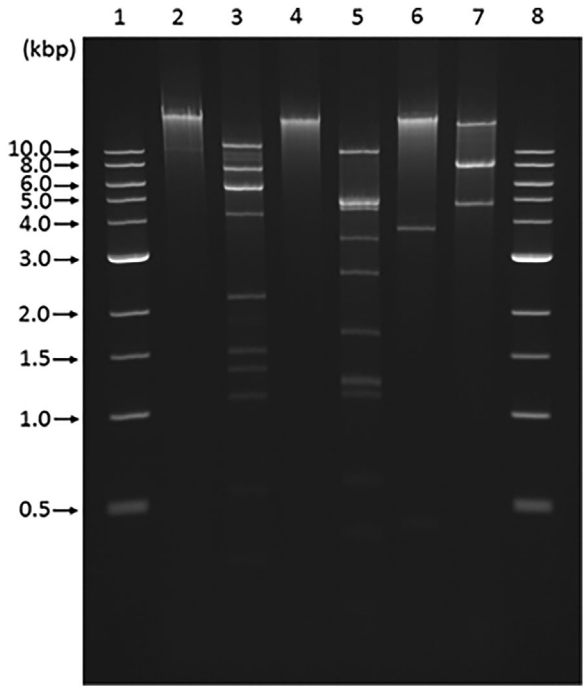

FIGURE 3 Digestion of genomic DNA of phage RsPod1EGY with six restriction enzymes, lanes 1 and 8 show standard molecular marker (1 kb), lanes 2, 3, 4, 5, 6, and 7 show restriction analysis of phage RsPod1EGY DNA with *Eco*RI, *Cla*I, *Not*I, *Acc*I, *Spe*I, and *Hind*III restriction enzymes, respectively

3.9 | *In vitro* bacteriolytic activity of RsPod1EGY phage

When the culture of *R. solanacearum* strain K3 was infected by phage RsPod1EGY with different MOI values, the bacterium showed a high susceptibility to phage infection. A noticeable gradual reduction in OD was observed with 1.0, 0.5, and 0.1 MOI, where the reduction was about 73, 82, and 90%, respectively, when compared to the untreated control, which reached 1.25 OD at 24 h (Fig. 6a).

3.10 | Virulence factors of *R. solanacearum* after phage treatment (surface exopolysaccharides)

Surface polysaccharide concentrations detected in *R. solanacearum* K3 culture infected with RsPod1EGY phage with different MOI values, showed a gradual reduction to undetectable limit 24 hr post infection (Fig. 6b).

3.11 | Biocontrol of *R. solanacearum* race 3 biovar 2 by treating with lytic bacteriophage RsPod1EGY under greenhouse conditions

Pretreatment of tomato seedlings with lytic bacteriophage RsPod1EGY under greenhouse conditions showed a suppressive effect on *R. solanacearum* race 3 biovar 2 phyllotype II sequevar 1. Wilt suppression was indicated by a 100% decrease in the wilt severity (i.e., no symptoms), as well as a decrease in population count of *R. solanacearum* to undetectable in the crown area of the tomato plants when compared to the control. The results shown in Fig. 7b indicated that tomato plants treated with RsPod1EGY phage were efficiently protected from wilting. Untreated tomato plants (without RsPod1EGY treatment) showed the first wilting symptoms 3 days post infection (dpi) while all four plants replicates showed total wilting 9 dpi. (Fig. 7a, b). All four RsPod1EGY-treated plants replicates did not show symptoms of wilting during the experimental period (Fig. 7a, b). AUDPC was estimated for the untreated tomato plants to be 265 (SE ± 9.5), while it was 0 for phage treated tomato plants. The average population count of *R. solanacearum* detected in tomato crown area by plating on SMSA media was estimated to be 1.3×10^7 for the control treatment without phage after 10 days from soil inoculation. Real time PCR assay has showed a significant difference in the population count of *R. solanacearum* in the DNA extracts of the tomato crown area between control treatment without phage and phage treated plants. No CFU of *R. solanacearum* were detected in the crown area extract of phage-treated plants, otherwise, *R. solanacearum* were detected in the non- phage treatment where, Ct value was 26 with typical amplification curve.

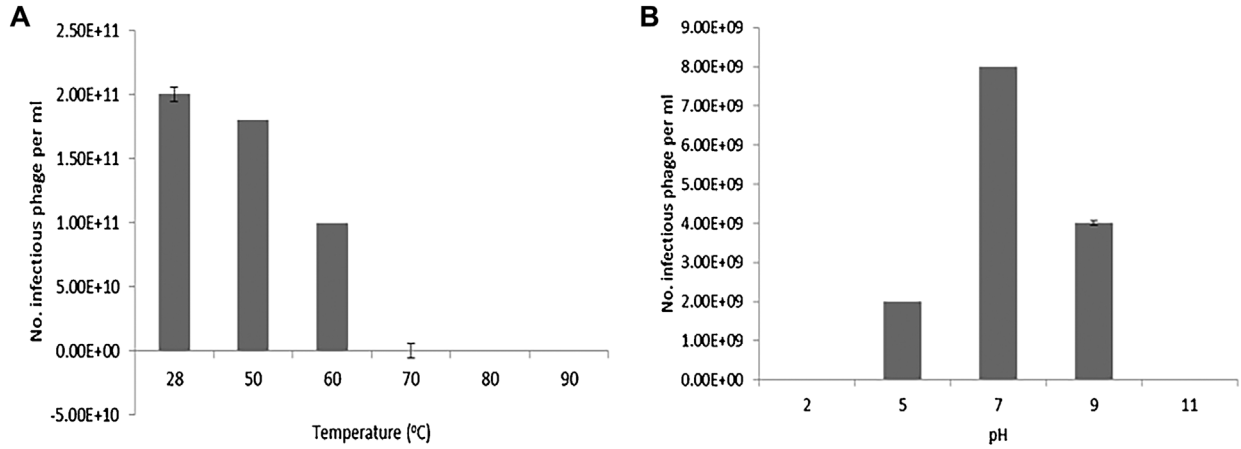

FIGURE 4 (A) Stability of phage RsPod1EGY held at different temperatures for 30 min and (B) stability of phage RsPod1EGY at different pH incubated for 3 h at 28 °C. All values represent means of three determinations ± SD. Error bars represent ± the standard deviation

4 | DISCUSSION

Our research represents an extensive characterization of new *R. solanacearum*-infecting phage isolated from potato plants and potato rhizosphere in the Nile Delta of Egypt. Previous studies have reported *R. solanacearum* phages in Egypt [34–37], but the current study mainly focused on the physical properties, protein characterization and restriction endonuclease analysis of the isolated phage and its efficacy to control bacterial wilt under greenhouse conditions. The phage appears to be specific to the only reported variant on potato in Egypt of *R. solanacearum* (phylotype IIa sequevar 1, race3 biovar 2). The results obtained in this study could contribute to control bacterial wilt disease as the phage was effective *in vitro* and under greenhouse conditions, however for field application further study might be effective. Different methods of control have been proposed against *R. solanacearum* including many cultural practices like crop rotation, soil solarization, planting time, using pathogen-free planting material and using tolerant cultivars [38]. Resistant potato genotype BP9, which is a somatic hybrid between *Solanum tuberosum* and *S. phureja*, successfully reduced bacterial wilt by 90–100% [39]. Unlike chemical bacteriocides, bacteriophages represent a natural component of the environment and impose no harm to human or the ecosystem. Bacteriophages often infect a narrow range of hosts, even at strain level. This feature can help be used when formulating mixtures of phages that target particular strains or species within a bacterial genus with minimum effect on non-target bacterial communities. Application of bacteriophage can be used as a control method against phytopathogenic bacteria, or to regulate bacterial communities associated to plants and affect their growth and yield [40]. A previous study showed that seed coating treatment of soybean with phage specific for *Bradyrhizobium japonicum* was able to improve nodulation by superior inoculum strain of rhizobium which increase nitrogen fixation activity significantly. Phage can be used to reduce the effect of undesirable strains of natural rhizobia in soil and improve nodulation by the desired rhizobia strains [40]. Specificity of phage is thought to refer to the presence of a specific set of binding sites expressed by host bacteria.

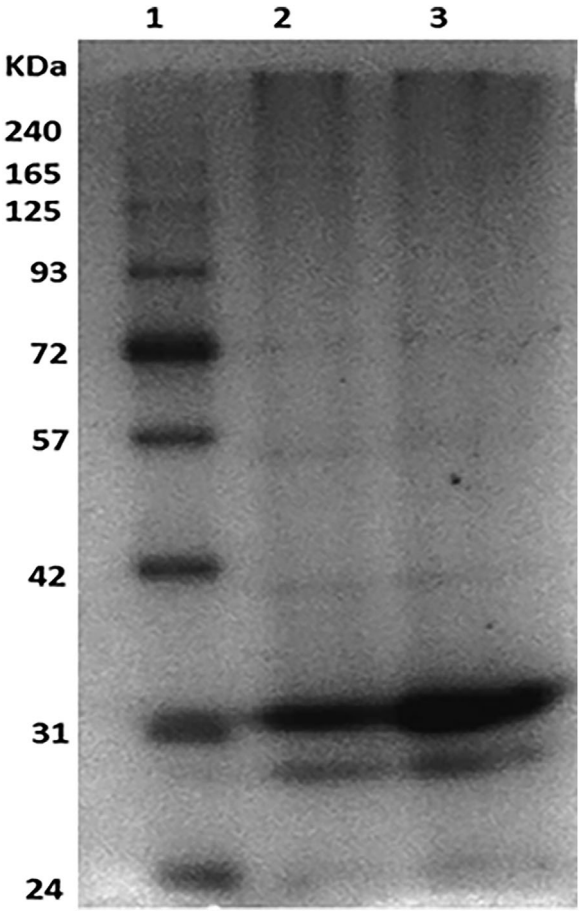

FIGURE 5 SDS–PAGE analysis of phage RsPod1EGY structural proteins. Lane 1, protein molecular mass markers, lanes 2 and 3 are duplicates of phage RsPod1EGY proteins, but with different running volume 15 and 20 µL, respectively

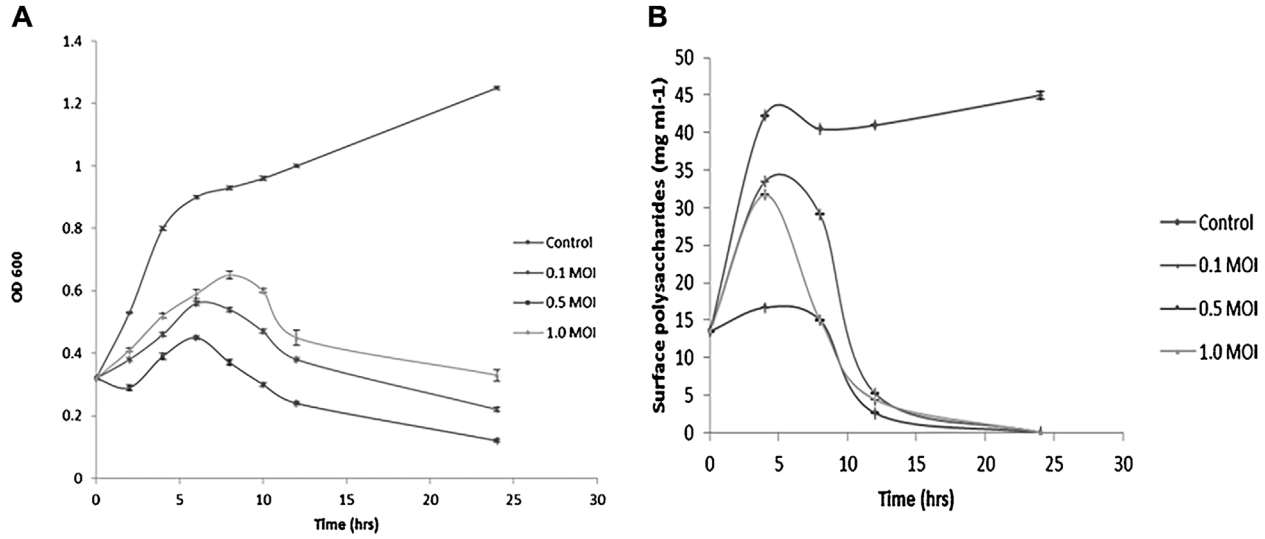

FIGURE 6 (A) The *in vitro* bacteriolytic activity of phage RsPod1EGY related to *R. solanacearum* growth and (B) to bacterial virulence factors (surface polysaccharides) at the exponential growth phase of the *R. solanacearum* at different values of MOI: 0, 0.1, 0.5, and 1.0. All values represent means of three determinations. Error bars represent the standard deviation

The lytic phage that was isolated from soil samples collected in the Nile Delta, Egypt by us was characterized as a podovirus based on its morphology and whole genome sequence reported in a separate study [41]. In Japan ϕRSB1, ϕRSB2, ϕRSB3 ϕRSJ2, and ϕRSJ5 podoviruses were previously isolated against *R. solanacearum* and identified by ref. [22,42,43]. Electron microscopic examination of *R. solanacearum* phage particles ϕRSB1, ϕRSB2, ϕRSB3, ϕRSJ2, and ϕRSJ5 showed a virion structure similar to T7-like phages that belong to Podoviridae family with short-tailed icosahedral, and head diameter of nearly 60 nm and short-thick tail ranging of 8–10 nm in length [43,44,45].

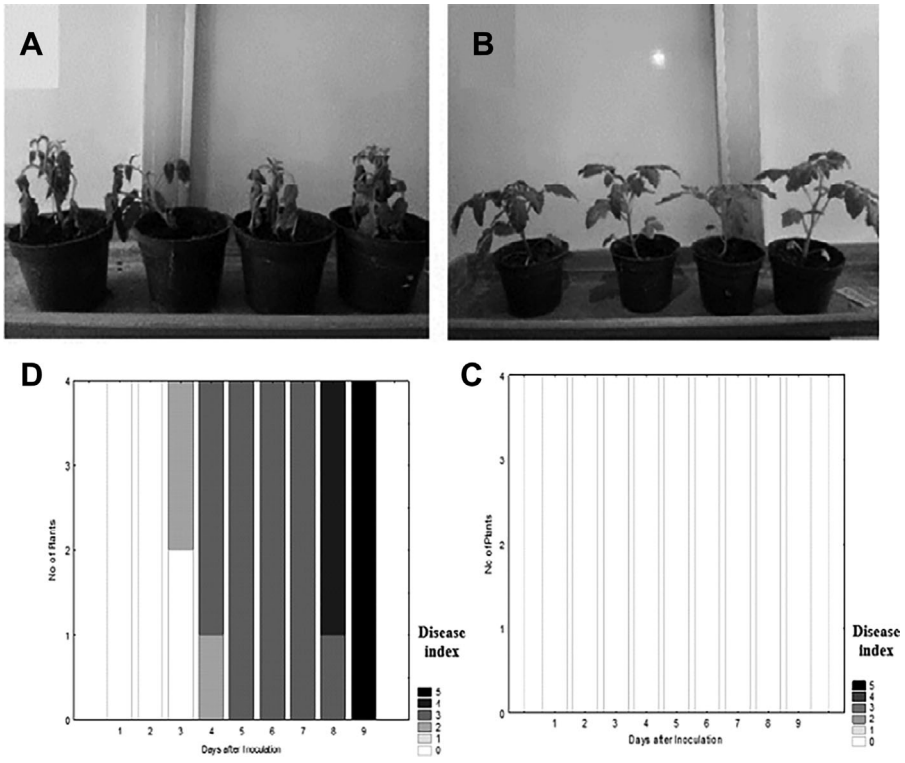

FIGURE 7 (A) Photo of untreated inoculated tomato plants showing bacterial wilt symptoms 9 days after artificial inoculation of the pathogen, (B) photo of phage treated inoculated tomato plants with no symptoms of bacterial wilt, (C) the disease incidence of bacterial wilt disease in tomato plants after treatment of lytic bacteriophage RsPod1EGY compared to (D) untreated plants grown under greenhouse conditions

RsPod1EGY phage has shown to belong to the Podoviridae family, order Caudovirales [41].

Phage RsPod1EGY has a high lytic activity, producing clear plaques with diameters ranging from 3.0 to 4.0 mm. Previous study reported that a phage, ϕRSB1 was isolated against *R. solanacearum* with a wide host range that exclusively follows a lytic cycle for replication, forming large plaques with a diameter of 1.0–1.5 cm [22]. A limited study on our phage host rang was performed only against the dominant phylotype in Egypt (phylotype IIa sequevar 1, race3 biovar 2) because other phylotypes are never reported before in Egyptian soil especially for potato crop. Till so far we found, RsPod1EGY phage infecting all tested strains of *R. solanacearum* rather than other tested plant pathogenic bacterial strains, indicating a possible high specificity of isolated phage. In this regard, a previous study [37] reported that, ϕRSB1 phage has wide host-range and infect more than two-thirds of the *R. solanacearum* strains tested, including strains with phylotypes I and IV, races 1, 3, and 4. While ϕRSB3 infected only five strains of the 21 *R. solanacearum* strains tested and ϕRSB2 infected 13 strains of the 21 strains tested. Many phages are interacting specifically with definite receptors present on the surface of the host bacterial cell, while will not be able to interact with receptor having different structures [46]. Generally, podoviruses such as T7-like phages are extremely lytic with no lysogenic cycle [47]. Such high lytic activity will allow them to be efficient biocontrol tools against bacterial diseases. The infection cycle of phage RsPod1EGY was typically 180 min for one round, with a burst size of 27–30 PFU per cell. In the case of ϕRSB2, phage infection happened with *R. solanacearum* strains (MAFF 730138), with an infection cycle of 60 min and a latent period of 40 min, giving a burst size of ca. 30 PFU/cell [43]. Phage characteristics such as heat and pH sensitivity are important factors for the application of the phage in different environments for biological control.

Temperature is a decisive factor for bacteriophage survivability [48]. Different phage strains show variable reaction toward heat and pH. Our results showed that the newly isolated phage RsPod1EGY has a high tolerance toward a broad range of temperature, ranging from 28 to 60 °C, but it became completely inactive at 70 °C. The phage revealed good pH stability toward variable pH values ranging from 5 to 9, with maximum stability at pH 7.0. These results are in agreement with earlier observations by ref. [49] that most phages can survive over a wide range of pH (5–9) in physiological conditions that keep the native structure and stability of virion units. The inactivity of the phage at lower pH value can be explained by denaturation of virion protein in acidic environments [50]. T7 phage lost almost all infectivity properties when incubated for 96 h in citrate or citrate–phosphate buffer with pH value lower than 4, while, at pH 3, the phage lost its activity after 1 h [51]. The genome size of phage RsPod1EGY was approximately 41 kb, similar to those of other podovirus phages against *R. solanacearum* ϕRSB1, ϕRSB2, ϕRSB3, ϕRSJ2, and ϕRSJ5 (40.4–44.6 kb) isolated from Japan [22,43]. The SDS–PAGE analysis confirmed closeness of RsPod1EGY to Podoviridae phage. The major head protein from RsPod1EGY podoviral phage was determined to have a size of 32 kDa, similar to the phage Syn5 which has 35 kDa major capsid protein [52]. Eight protein bands were observed for phage RsPod1EGY on SDS-PAGE while, *R. solanacearum* phage ϕRSB1 showed nine protein bands [22]. When RsPod1EGY phage was used to treat *R. solanacearum* culture suspension, the turbidity (Optical Density OD) of the suspension decreased significantly, indicating lysis of bacterial cells. The activity of phage that inhibits *R. solanacearum* growth and lyses its cell, indicates its possible suitability of using as biocontrol of *R. solanacearum* pathogen.

Bacterial polysaccharides serve as the primary receptors for phage adsorption into bacterial host cell, afterward the phage degrades polysaccharide receptors to complete its lytic cycle. When *R. solanacearum* was infected with phage RsPod1EGY at different MOI values, bacterial surface polysaccharides were apparently decreased to undetectable after 24 h of culture incubation. It demonstrates that, RsPod1EGY phage efficiently infect host cells with a high lytic activity. The lytic phage suggested to encode depolymerase enzymes, which support capable anti-bacterial treatment [53]. Phage RsPod1EGY has a good lytic activity on its bacterial host in infested soil of tomato plants grown under greenhouse conditions. Phage RsPod1EGY protected tomato plants from wilting caused by *R. solanacearum* when used as seedlings pre-treatment. In a previous study in Japan [12], when tomato plants were treated with phage ϕRSL1 under greenhouse conditions, they were protected from bacterial wilt throughout the experiment period, while all untreated tomato wilted within 18 days post-infection.

In conclusion, this study suggests that the isolated phage RsPod1EGY from the Egyptian soil is a host specific, novel lytic podovirus with a wide temperature range up to 60 °C and stable over a range of pH (5–9). It also, has a high lytic activity against *R. solanacearum* phylotype IIa sequevar I, race3 biovar 2 *in vitro* and *in vivo* under greenhouse conditions. Moreover, it succeeded in total elimination of *R. solanacearum* surface polysaccharides. Its application to tomato pots infested with the pathogen completely suppressed the bacterial wilt disease under greenhouse conditions, and may therefore possibly be used as an effective biocontrol agent against *R. solanacearum*. Further studies with this promising phage under field conditions using potato are foreseen in the future to confirm the results of the greenhouse tomato trial for our promising phage.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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