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Research Paper

Isolation of *Dickeya dadantii* strains from potato disease and biocontrol by their bacteriophages

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Abstract

One of the most economically important bacterial pathogens of plants and plant products is *Dickeya dadantii*. This bacterium causes soft rot disease in tubers and other parts of the potato and other plants of the *Solanaceae* family. The application of restricted host range bacteriophages as biocontrol agents has recently gained widespread interest. This study purposed to isolate the infectious agent of the potato and evaluate its biocontrol by bacteriophages. Two phytopathogenic strains were isolated from infected potatoes, identified based on biochemical and 16S rRNA gene sequencing, and submitted to GenBank as *D. dadantii* strain pis3 (accession no. HQ423668) and *D. dadantii* strain sip4 (accession no. HQ423669). Their bacteriophages were isolated from Caspian Sea water by enriching the water filtrate with *D. dadantii* strains as hosts using spot or overlay methods. On the basis of morphotypes, the isolated bacteriophages were identified as members of the *Myoviridae* and *Siphoviridae* families and could inhibit the growth of antibiotic resistant *D. dadantii* strains in culture medium. Moreover, in *Dickeya* infected plants treated with bacteriophage, no disease progression was detected. No significant difference was seen between phage-treated and control plants. Thus, isolated bacteriophages can be suggested for the biocontrol of plant disease caused by *Dickeya* strains.

Key words: *Dickeya dadantii*, soft rot, bacteriophage, antibiotic resistance, biocontrol.

Introduction

Each year, bacterial diseases cause significant loss of plant productions during cultivation and storage. Some members of the *Enterobacteriaceae* family, such as *Dickeya dadantii* sp. (Samson *et al.*, 2005), formerly known as *Erwinia chrysanthemi* (Hauben *et al.*, 1998), and *Pectobacterium chrysanthemi,* cause soft rot and black age disease in a wide range of crop plants such as sorghum, urn plant, sugarcane, rice, poinsettia, taro, nephthytis, carnation, African violet, pineapple, Aglaonema, philodendron, Dieffenbachia, orchid, cyclamen, tomato, *Geranium*, begonia, dahlia, corn, sweet potato, banana, gladiolus, potato, and carrot (Dickey, 1979; McFadden, 1958). These are Gram-negative, oxidase negative bacteria which grow at 39 °C. Samson *et al.* (2005) placed *P. chrysanthemi* in the genus *Dickeya* spp. and reclassified *Erwinia* (*Pectobacterium*) into the following six *Dickeya* species: *D. chrysanthemi*, *D. paradisiacal*, *D. dadantii*, *D. dianthicola*, *D. dieffenbachiae,* and *D. zeae* (Samson *et al.*, 2005). In Finland, *Pectobacterium* and *Dickeya* sp. were isolated from infected potato stems and tubers and identified by sequencing as *P. atrosepticum*, *P. carotovorum,* and pathogens in the genus *Dickeya*. Furthermore, *Dickeya* strains were isolated from river water samples (Laurila *et al.*, 2008). *E. chrysanthemi* disease on potato was reported in the 1980s, and *D. dadantii* sp. disease on potato was reported in 2008 (Tsror *et al.*, 2009). Several reports have indicated that *D. dadantii* could cause soft rot disease in potato seeds (Czajkowski *et al.*, 2009; Ngadze *et al.*, 2010). Hedjarood first isolated *E. chrysanthemi* on cyclamen in Iran in 1967.

The microbial agent of Erwinias soft rot disease is widespread in the environment; thus, controlling it is difficult. Several chemical compounds including copper, formaldehyde, 8-hydroxyquinoline, anthium dioxide, chlorine dioxide, gluytaraldehyde, benzalkonium chloride, cetalkonium chloride, and others are used to control Erwinias soft

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rot disease, but they are not very effective on this bacterium (Letal, 1977; Lund and Lyon, 1975; Wyatt and Lund, 1981). Potato diseases have been effectively controlled with antibiotics such as streptomycin, tetracycline, and vancomycin, but the development of antibiotic resistance in soil pathogens has limited their application (Gnanamanickam, 2007).

Recently, bacteriophages have been used as biocontrol agents to control pathogenic bacteria (Górski and Weber-Dabrowska, 2005; Jones *et al.*, 2007; Smith *et al.*, 1987; Verthé *et al.*, 2004). The advantages of bacteriophages over other biocontrol agents include specificity to their bacterial hosts and non-pathogenicity for humans and animals (Czajkowski *et al.*, 2013).

Not much information exists about *D. dadantii* or *E. chrysanthemi* lytic bacteriophages or their use in the biocontrol of plant disease. However, some studies have reported the use of bacteriophages against *Erwinia* and *Dickeya* spp. For example, Czajkowski *et al.* (2013) isolated lytic bacteriophages infecting *Dickeya* spp. Biovar 3, and used them to protect potato tuber tissue from symptoms of *Dickeya* infection. Fourteen bacteriophages of *E. carotovora* subsp. *carotovora* were isolated from samples of fertilizer solutions by Ravensdale *et al.* (2007), who were able to reduce the incidence of soft rot symptoms.

The current study aimed to isolate and identify new specific native bacteriophages against *D. dadantii* as potential agents for the biocontrol of plant disease caused by *Dickeya*.

Materials and Methods

D. dadantii isolation

Potato tuber and stem samples were collected from potato farms in Flavarjan, Isfahan, Iran (GPS coordinates of sampling locations include: 1: latitude: 32°33'33.26" N and longitude: 51°29'11.10" E; 2: latitude: 32°33'33.06" N and longitude: 51°29'10.62" E; 3: latitude: 32°32'29.53" N and longitude: 51°30'6.33" E). Samples were stored at 4 °C immediately after collection. To isolate bacteria, the external surface of the infected tubers and stems were disinfected in two steps: first with 70% ethanol, and then with 0.1% $HgCl_2$. Finally, they were rinsed 4-5 times with sterile distilled water. Small amounts of tuber and stem samples were taken from the margin of healthy and diseased tissue and plated on Nutrient Agar (NA, Scharlou, Spain) and Brain Heart Infusion Agar (BHI, Merck, Germany) media (Laurila *et al.*, 2008). After incubation at 28 °C for 24 hours, distinct colonies were purified by repeated subculturing on NA and BHI agar media.

Biochemical and molecular identification of isolated bacteria

Primary identification was performed according to Brenner *et al.* (2005). For molecular identification, the isolated bacteria were cultured on NB medium at 28 °C for 48 hours. Genomic DNA was extracted by a commercial extraction kit (DNA extraction kit, Cinnagen, Iran) and amplified by two universal primers including RW01 as forward primer (5'AAC TGG AGG AAG GTG GGG AT3') and DG74 as reverse primer (5'AGG AGG TGA TCC AAC CGC A3') (TAG Copenhagen, Denmark) (Leong and Greisen, 1993). The nucleotides position of RW01 forward primer is 1172-1191 and of DG74 reverse primer is 1540-1522 of the DNA sequence coding for 16S ribosomal RNA of *E. coli* (GenBank accession number: E05133). PCR mixture for amplification of the 16S rRNA gene contained 2.5 µL 10X PCR buffer, 0.7 µL $MgCl_2$, 0.5 µLdNTP, 20 pM of forward and reverse primers, 2 µL DNA template, and 0.5 µL DNA Taq polymerase (Cinnagen, Iran). PCR conditions were as follows: initial denaturation at 95°C for 5 min (1 cycle), denaturation at 94 °C for 45 seconds, annealing at 54 °C for 30 seconds, extension at 72 °C for 45 seconds in 30 cycles, and final extension at 72 °C for 5 min. The PCR products (370 bp length) were sequenced by Macrogen, South Korea.

Antibiotics susceptibility test

The disk diffusion method was used for the antibiotic susceptibility test in Muller Hinton Agar (MHA, Merck, Germany). Zones of inhibition were measured (mm) and susceptibility was determined by standard tables of antibiogram (CLSI 2010).

Isolation of bacteriophages

In previous and parallel studies, it was hypothesized that Persian Gulf water and Caspian Sea water have great potential for bacteriophage isolation, because some rivers and agricultural wastes, such as biological matter (bacterial, viral, and fungal pathogens), sloped to the Caspian Sea shorelines. Based on this hypothesis, Caspian Sea water was used as a source for bacteriophage sampling. Samples of Caspian Sea water were collected from a depth of 50-100 cm along the shorelines of Kiashahr, Guilan, Iran and stored under sterile conditions at 4 °C. GPS coordinates of sampling locations include: 1: latitude: 37°26'27.33" N and longitude: 49°57'54.45" E; 2: latitude: 37°26'26.66" N and longitude: 49°57'55.15" E; 3: latitude: 37°26'25.22" N and longitude: 49°57'50.44" E; 4: latitude: 37°28'16.64" N and longitude: 49°56'22.07" E; 5: latitude: 37°28'12.59" N and longitude: 49°56'26.38" E. Samples were centrifuged (12,000x *g*, 10 min), and the supernatant was filtrated through a 0.22 micrometer cellulose acetate membrane syringe filter (Orange Scientific, Belgium). Then, glycine buffer (1% w/v-Sigma, Germany) was added to the filtered samples for preservation of bacteriophages, and the samples were stored at 4 °C. A loop full of isolated bacteria was cultured in BHI broth medium as host, then after 2 hours pre-incubation, 1,000 µL of phage-filtered sample was added and incubated at 28 °C overnight. The bacterial cul-

ture was then centrifuged at 12,000x *g* for 10 min and filtrated through 0.22 µ filters (Gill *et al.*, 2003; Ritchie and Klos, 1977). The soft-agar overlayer method was used for bacteriophage plaque formation (Clokie and Kropinski, 2009). Diluted bacteriophage filtrates were added to a tube of warm overlay NA medium (0.7%), enriched with 100 µL of *D. dadantii* bacterial culture as host, plated on sublayer (NA medium), and incubated at 28 °C for 12 hours. This procedure was repeated 5-7 times to purify phages isolated from single plaques.

Preparation of high titer bacteriophage

Phages were propagated on isolated *D. dadantii* strains. First the phage originating from a single plaque was mass streaked on the lawn of the propagating bacterium on a soft NA (0.7%) with a sterile toothpick. Following overnight incubation at 28 °C, the phages were eluted with 5 mL sterilized glycine buffer (1%), and the resulting phage suspensions (10^{10} PFU/mL) were used to prepare high-titer suspensions as follows: 100 mL Nutrient Broth (NB) medium (Gill *et al.*, 2003; Ritchie and Klos, 1977) was inoculated by the propagating bacterium to 10^8 cells/mL final concentrations and 10^7 PFU/mL from phage. After a 15-min preincubation time, the cultures were placed in a rotary shaker (50 rpm) for overnight incubation at 28 °C. Bacterial debris was eliminated from the lysates by further centrifugation (12000x *g*, 10 min), then either glycine buffer (1% v/w) or chloroform-treated buffer was added for preservation of the bacteriophages, and they were stored at 4 °C. All controls were stored under the same conditions in sterile double-distilled water without using glycine or chloroform. Bacteriophages were titrated using the direct plate plaque assay based on the method introduced by Mazzocco *et al.* (2009) and calculated as follows:

Average number of plaques x 5 x reciprocal of counted dilution = PFU/mL

Growth curve of isolated *Dickeya* with bacteriophage

The purified colonies of isolated strains were cultured in NB medium (28 °C, 120 rpm, overnight). Two percent (v/v) of the enriched bacterial culture was inoculated to 250 mL NB medium in 1 L flasks and incubated on a rotary shaker (120 rpm) for 30 hours. The optical densities of culture were measured at 2-hour intervals. The experiment was repeated using the same conditions except that the bacteriophage suspension (3x 10^9 PFU/mL) was added to the bacterial culture for analysis of bacterial lysis activity of the isolated phages.

Electron microscopy

High titer bacteriophage suspensions were transferred onto copper-coated grids with formvare for 2 min. The additional suspension was dried using Watman filter paper, and the grade was stained by 1% uranyl acetate for 30 seconds. The grids were viewed using Philips CM10 transmission electron microscopy.

Antibacterial effects of phages on *Dickeya dadantii*-infected *Geranium* spp. at laboratory scale

The two *Siphoviridae*- and *Myoviridae*-related phages were used separately to control *D. dadantii* sip4 pathogenicity in cranesbill (*Geranium* spp.). Bacterial suspension grown in an NB medium at logarithmic growth phase (A_{600nm} = 0.6) was washed with sterile deionized water and resuspended in the same water to 5x10^8 cells/mL. Then it was used to create lesions in *Geranium* being sprayed onto leaves (group 1). Similar to group 1 and two days before spraying the bacterial suspension onto the leaves, 3x10^9 PFU/mL of *Siphoviridae*- and *Myoviridae*-related bacteriophages were sprayed onto leaves in experimental group 2. Plants in group 3 were tested without any bacteria or bacteriophages treatments. The 3 groups of plants were stored in greenhouse conditions at 32 °C and 80-90% humidity. This experimental procedure was repeated nine times under the same conditions, and the results were examined using Mann-Whitney U and Kruskal-Wallis tests. All statistical analyses were carried out at $\alpha = 0.05$ significance level.

Results

Dickeya dadantii isolation and identification

The bacteria isolated from infected potatoes (*Solanum tuberosum*) were identified by biochemical tests for the *Erwinia* group (Table 1). In molecular analyses, the amplified 16S rRNA genes of two isolated bacterial strains

Table 1 - The results of morphological and biochemical characteristics of *Dickeya* spp.

Test	*D. dadantii* pis3	*D. dadantii* sip4
Shape	R	R
Motility	+	+
Aerobic growth	+	+
Anaerobic growth	-	+/-
Catalase	+	+
Oxidase	-	-
Pink pigment	-	-
Growth at 36 °C	+	+
Urease	-	-
Pectate lysate	+	+
Gelatin hydrolysis	+	+
Growth in 5%NaCl	+	+
Lesitinase	+	-
Erythromycin*	+	-

R: Rod shape; (+/-): not clear; *sensitivity to erythromycin; (+) positive; (-) negative.

were identified using the megablast program of NCBI (www.NCBI.nlm.nih.gov). Due to the high homology of the amplified ribosomal DNA sequences with *D. dadantii*, two bacterial strains isolated in this experiment were submitted to GenBank as *D. dadantii* pis3 (GenBank accession number HQ423668) and *D. dadantii* sip4 (GenBank accession number HQ423669).

Antibiotic sensitivity

The results of the antibiotic susceptibility test for isolated *Dickeya* are shown in Table 2. The isolated strain pis3 was sensitive to amikacin, siprofloxacillin, and oxytetracyclin. Strain pis3 was also sensitive to erythromycin, but strain sip4 was resistant (Table 2).

Isolation of bacteriophages and assessment of plaque morphology

The bacteriophage produced two various types of plaque morphology on the *D. dadantii* lawn culture: large plaques with 5 mm diameters and opaque halos in their margins and small plaques with 1-2 mm diameters and opaque halos (Figure 1).

Growth curve of isolated *Dickeya* with bacteriophage

The growth rates of the isolated bacteria with and without bacteriophage are shown in Figure 2. In the presence of bacteriophage, bacteria growth was inhibited for 17 hours, after which, it was initiated and increased.

Transmission electron microscopy

Two types of bacteriophage were identified based on their morphology. Bacteriophages with regular hexagonal head of 90-100 nm length, 25-30 nm width, and 120 nm long tails were identified as the *Siphoviridae* family (Figure 3a) and bacteriophages with hexagonal heads (approximately 100 nm length and 50-60 nm width) and 90-100 nm long tails were identified as the *Myoviridae* family (Figure 3b).

Bacteriophage enumeration in preservative buffer

The obtained phage suspensions had titers of up to 1.9 x 10^{10} PFU/mL. The bacteriophage was enumerated both pre- and post-incubation at 4 °C for 8 months. All results indicated that glycine could preserve bacteriophage particles for up to 8 months at 4 °C in good conditions with no apparent change in phage titer, while for phages preserved with chloroform-treated buffer, and double distilled water, bacteriophage titration decreased (Table 3).

Table 2 - Antibiotic sensitivity test of *Dickeya dadantii* strains pis3 and sip4.

Antibiotics	g per disc	Sensitivity	
		Strain pis3	Strain sip4
Oxytetracyclin	30	S	I
Carbenicillin	100	R	R
Tobramycine	10	R	R
Erythromycin	15	S	R
Nalidixic acid	30	I	S
Vankomycin	30	R	R
Cloxacillin	5	R	R
Chloramphonicle	30	I	R
Clindamycin	2	R	R
Ceftisoxim	30	R	R
Siprofloxacillin	5	S	I
Amikacin	30	S	S

R = Resistant; I = Intermediate; S = Sensitive.

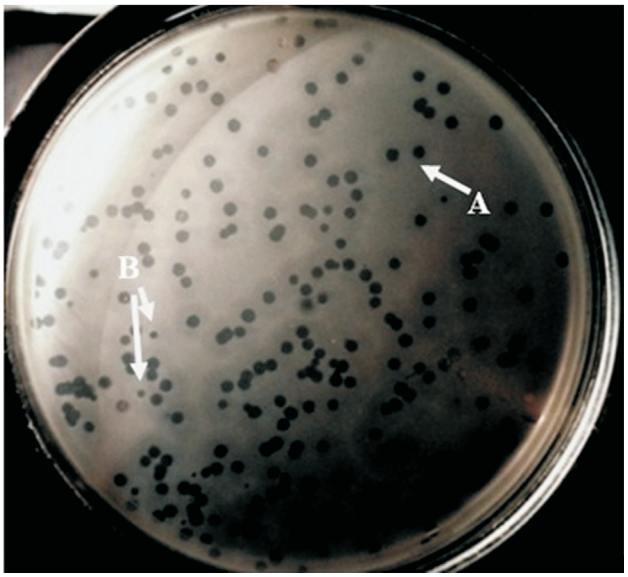

Figure 1 - Plaques formation in agar medium. The isolated *Dickeya* propagated on soft nutrient yeast agar medium, and the phage suspension spread on the medium. A) plaques up to 4-5 millimeter diameter (*Myoviridae* phage), and B) plaque up to 2-3 millimeter diameter (*Siphoviridae* phage).

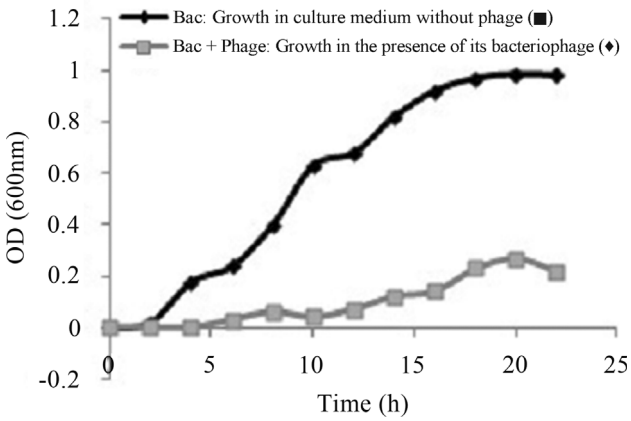

Figure 2 - Growth curve of *Dickeya dadantii*.

Table 3 - Titers of two isolated bacteriophages (PFU/mL) in glycine solution and pure water (DDW).

Isolated bacteriophage	Solution	pre incubation	After 4 months	After 8 months
Myoviridae	Glycine	2.2×10^{10}	2.1×10^{10}	1.97×10^{10}
	DDW	2.2×10^{10}	0.5×10^{10}	-
Siphoviridae	Glycine	1.77×10^{10}	1.68×10^{10}	1.63×10^{10}
	DDW	1.77×10^{10}	0.65×10^{10}	$\leq 0.1 \times 10^{10}$

(-) Without plaque, DDW (double distilled water).

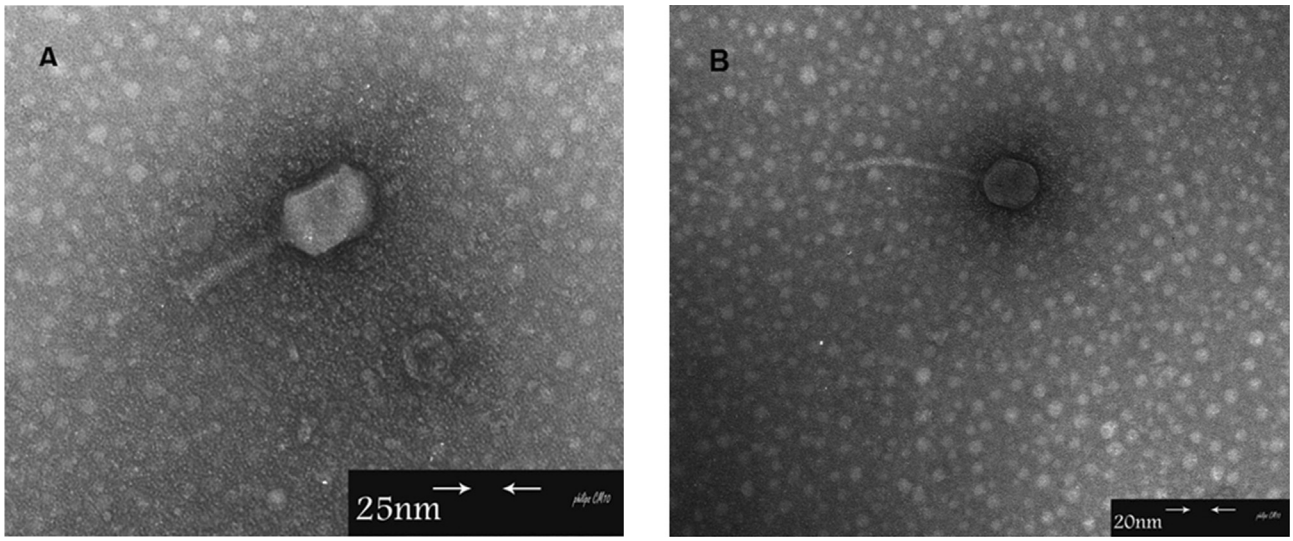

Figure 3 - Electron micrograph of *Dickeya dadantii* phages: (a) *Myoviridae* family, (b) *Siphoviridae* family.

D. dadantii pathogenesis reduced by its bacteriophage

After 5 days of incubation, *Geranium* spp. with the bacterial suspension in group 1 showed several symptoms of lesions, such as chlorosis and necrosis on leaves and stems. After 5 days, the leaves had rotted, and plants infected with *D. dadantii* sip4 were completely rotten. In the second group of plants that were treated with bacterial and bacteriophage suspensions, only 3 of the 27 tested plants treated with *Myoviridae*-related bacteriophage faded completely after five days. Other treated geraniums in this group grew without any symptoms of disease. Data was analyzed with the Kruskal-Wallis test, and four groups within the groups were significantly different ($p = 0.00$). Moreover, the differences between bacteria/phages ($p = 0.00$), bacteria/control ($p = 0.00$), bacteria/phage siph ($p = 0.00$) were statistically significant; between phage/control treatment, phage myo/control ($p = 0.258$), phage myo/phage siph ($p = 0.863$), and control/phage siph ($p = 0.222$), the data for phages and control treatment showed no statistical differences (Figure 4).

Discussion

D. dadantii causes soft rot disease in plants of the *Solanaceae* family, including the potato (Czajkowski *et al.*, 2009; Laurila *et al.*, 2008; Ngadze *et al.*, 2010; Tsror *et al.*, 2009). In the current study, two strains of *D. dadantii* were isolated from infected potato tissue for the first time in Iran. Also isolated were two lytic bacteriophages of the *Myoviridae* and *Siphoviridae* families from water of the Caspian Sea, located in northern Iran, against isolated strains of *D. dadantii* from infected potatoes from central Iran (Isfahan province). Adriaenssens *et al*. identified two bacteriophages, LIMEstone 1 and LIMEstone 2, against *D. solani* belonging to *Myoviridae* which had icosahedral heads of 91.4 nm and tail dimensions of 113.8x17 nm (Adriaenssens *et al.*, 2012).

Schenabel and Jones (2001) reported five bacteriophages of *E. amylovora* isolated from apple orchards that belonged to *Myoviridae*. Czajkowski *et al*. isolated nine bacteriophages infecting *Dickeya* spp. biovar 3 from soil of distinct geographical locations in Poland, which were morphologically related to the order Caudovirales, family *Myoviridae* (Czajkowski *et al.*, 2013). Gill *et al*. isolated 42 bacteriophages against *E. amylovora* from pear and apple

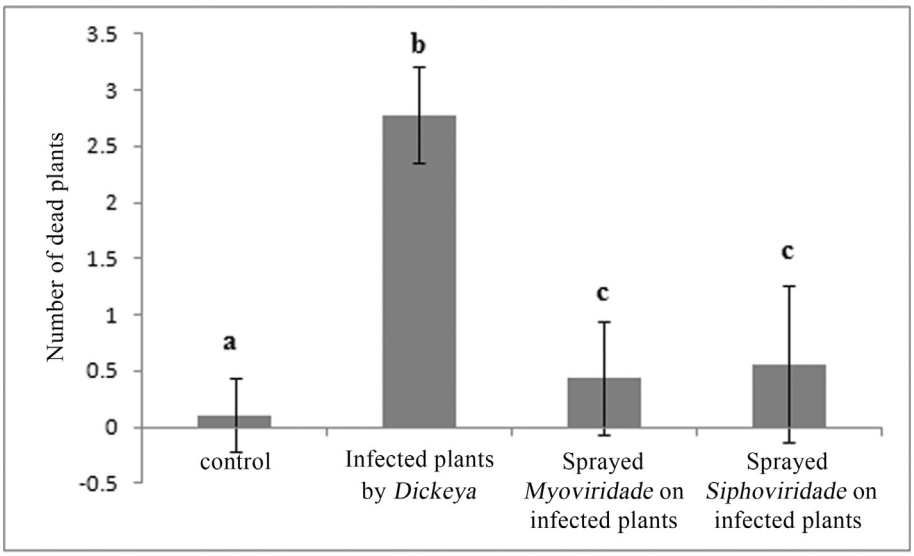

Figure 4 - Cranesbill (*Geranium* spp.) plants infected by 5 x 10^8 cells/mL of *Dickeya dadantii* sip4 and treated by 3 x 10^9 PFU/mL of *Siphoviridae* and *Myoviridae* related bacteriophages, plant in control group treated with DDW, and plants in bacteria group inoculated with 5 x 10^8 cells/mL of *Dickeya dadantii* sip4.

that were from the *Myoviridae* family and were smaller than ones isolated in the current study (Gill *et al.*, 2003). Only Kishko *et al*. seem to have reported the isolation of bacteriophages of *Siphoviridae* infecting *E. carotovora* (Kishko *et al.*, 1983). This study isolated two members of *Myoviridae* and *Siphoviridae* bacteriophage infecting *D. dadantii* strains from rotting potato tubers for the first time in Iran.

In many studies the use of a few drops of chloroform in the buffer is suggested for the preservation of various bacteriophages at 4 °C (Clokie and Kropinski, 2009). The isolated phages that were identified as the *Siphoviridae* and *Myoviridae* families were unstable in chloroform. Therefore, for bacteriophage preservation, the isolated phages in this study were found to be stable in the solutions made with 1% (w/v) glycine buffer rather than in buffer solutions with chloroform or in double distilled water. Glycine buffer could preserve phages with no significant change in the titer for up to eight months at 4 °C.

Isolated lytic bacteriophages from the Caspian Sea samples were able to significantly reduce population of isolated *Dickeya* strains from central Iran in the culture media. In first phage titration of Caspian Sea water samples without phage enrichment, approximately 271 plaque units were observed on *Dickeya* strains. This massive plaque forming unit (PFU) of bacteriophage indicated that Caspian Sea water has great potential for phage therapy in this case. This observation indicates that the sampling regions (Guilan province and Kiashahr farmlands and rivers) are contaminated and infected with *Dickeya* spp. or other related phytopathogens.

Erwinia cannot be controlled by specific bactericides (like other phytopathogenic bacteria) (Gnanamanickam, 2007). The incidence of antibiotic resistance may increase among bacteria due to the use of antibiotics in treating plant diseases, and this may be an origin of antibiotic-resistance genes in agroecosystems and the transfer of these genes to human pathogenic bacteria. Other compounds such as copper- and sulfur-containing formulations are not very effective and cause environmental pollution (Gnanamanickam, 2007). In this study, the isolated bacteria were resistant to most antibiotics but were lysed completely by isolated bacteriophages; so, these bacteriophages are capable of biocontrolling plant disease caused by specific bacterial hosts.

Data from this study showed that *D. dadantii* strains caused necrosis, soft rot, and black age disease in *Geranium* spp. in a few days. However, the *Dickeya*-infected plants (*Geranium* spp.) which were treated with specific bacteriophages showed no symptoms of disease in comparison with infected non-treated plants. Regarding the resistance of the isolated bacteria to most broad spectrum antibiotics, the use of the bacteriophages for controlling potato disease could be more effective in eliminating the targeted bacterial pathogens. Moreover, phages could be safe substitutes for toxic chemical agents such as antibiotics or other bactericidal agents in the control of plant diseases. However, more research with plants is needed to extend the findings of the current study regarding the application of biocontrol agents such as bacteriophage in agroecosystems.

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