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Biocontrol of *Pectobacterium carotovorum* subsp. *carotovorum* Using Bacteriophage PP1

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Pectobacterium carotovorum subsp. *carotovorum* (formerly *Erwinia carotovora* subsp. *carotovora*) is a plant pathogen that causes soft rot and stem rot diseases in several crops, including Chinese cabbage, potato, and tomato. To control this bacterium, we isolated a bacteriophage, PP1, with lytic activity against *P. carotovorum* subsp. *carotovorum*. Transmission electron microscopy revealed that the PP1 phage belongs to the *Podoviridae* family of the order *Caudovirales*, which exhibit icosahedral heads and short non-contractile tails. PP1 phage showed high specificity for *P. carotovorum* subsp. *carotovorum*, and several bacteria belonging to different species and phyla were resistant to PP1. This phage showed rapid and strong lytic activity against its host bacteria in liquid medium and was stable over a broad range of pH values. Disease caused by *P. carotovorum* subsp. *carotovorum* was significantly reduced by PP1 treatment. Overall, PP1 bacteriophage effectively controls *P. carotovorum* subsp. *carotovorum*.

Keywords: PP1 bacteriophage, *Pectobacterium carotovorum* subsp. *carotovorum*, biocontrol, soft rot disease

Introduction

Pectobacterium carotovorum subsp. *carotovorum* (formerly *Erwinia carotovora* subsp. *carotovora*) is a Gram-negative phytopathogen responsible for soft rot disease, wilt, or blackleg in various crops, by producing plant cell wall degrading enzymes that are actively secreted by the bacterium. Several important crops, such as Chinese cabbage and potato, have been attacked by this pathogen, and large economic losses resulting from significant yield reductions in the field, in transit, and during storage have occurred [11, 24, 27, 34]. However, effective control methods have not yet been developed.

Various strategies have been developed to control plant diseases, such as chemical antibiotics and copper, which have been used for many years [6, 25]. In addition, disease management involving cultural practices, plant activators, and plant resistance genes are extensively used to control various bacterial diseases. However, copper resistance has been reported in many bacterial pathogens, few effective bactericides have been developed, and plasmid-encoded antibiotic resistance genes are common [2, 6, 21, 31, 32]. Thus, novel strategies for the control of bacterial diseases are required. Particular attention has been paid to biologically based strategies, such as bacteriocins or bacteriophages [4, 17]. Recently, several bacteriocins have been developed, including a novel bacteriocin, carocin D, which was isolated from *P. carotovorum* subsp. *carotovorum* Pcc21 and used to control soft rot disease [29]. However, another environmentally friendly biological control agent, bacteriophage, has not been applied to the control of soft rot disease.

Bacteriophages are viruses that specifically infect bacteria. Typically, upon infection with a virulent phage, the bacterium is lysed and numerous progeny phages are released and infect neighboring bacteria. Therefore, the phage is amplified followed by bacterial lysis [9], which is an advantage of use of bacteriophages over other treatments. In addition, the lytic properties of bacteriophages are bactericidal rather

than bacteriostatic, and they are effective against antibiotic- or heavy-metal-resistant bacteria, and have high target specificity, and in some cases biofilm disruption activity [9, 13]. Bacteriophages are natural components of the biosphere and nontoxic to the eukaryotic cell, and their preparation is easy and inexpensive [18]. These characteristics suggest bacteriophages to be promising biocontrol agents. Bacteriophages specific for *P. carotovorum* subsp. *carotovorum* have been isolated from diseased plant materials and their associated soils, fertilizer solutions, cull piles, and sewage. Bacteriophages are isolated from these locations at lower frequencies and have narrower host ranges than phages that infect other Enterobacteriaceae [7, 12, 16, 27]. We reported previously the isolation of diverse bacteriophages collected from different areas of Korea [16]. Based on previous bacteriophage collection data, we characterized the lytic bacteriophage PP1 in terms of its efficacy as a biocontrol agent for *P. carotovorum* subsp. *carotovorum*. The bacteriophage was characterized both morphologically and phenotypically, and its antibacterial activity was confirmed *in vitro* and *in vivo*.

Materials and Methods

Bacteriophage Isolation

Soil samples were collected from Chinese cabbage fields in Pyeongchang, South Korea, which had been damaged by soft rot disease. The bacteriophage was isolated as described previously, with minor modifications [19]. Briefly, 5 g soil sample was homogenized with 5 ml of buffer (10 mM $MgSO_4$). After the addition of 100 µl of an overnight culture of *P. carotovorum* subsp. *carotovorum*, initial enrichment was performed for 24 h at 28°C. After centrifugation, the supernatant was filtered through 0.22-µm-pore-size filters (Sartorius, Germany). Ten-fold serial dilutions of these filtrates were used in a spotting assay against *P. carotovorum* subsp. *carotovorum* to confirm the presence of bacteriophage.

Propagation and Purification of Bacteriophage PP1

Bacteriophage was purified using five-time repeated overlay assays by picking plaques and eluting in sodium chloride-magnesium sulfate (SM) buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgSO_4$), as described previously [20]. *P. carotovorum* subsp. *carotovorum* Pcc3 was employed to propagate the bacteriophage. Bacteriophage particles were precipitated by treatment with polyethylene glycol (PEG) 6,000 (Sigma, USA) and purified using CsCl density-gradient ultracentrifugation (78,500 ×*g*, 2 h, 4°C). After dialysis, the phage stock was stored in glass tubes at 4°C.

Bacteriophage Morphology Assessment by Transmission Electron Microscopy

Solution containing bacteriophage was placed on carbon-coated copper grids, and 2% aqueous uranyl acetate (pH 4.0) was added for 20 s to negatively stain the phage particles. Phages were examined by transmission electron microscopy (TEM; LEO 912AB transmission electron microscope; Carl Zeiss), and images were scanned with a Proscan 1,024 × 1,024 pixel charge-coupled device camera. Phages were classified according to the guidelines of the International Committee on Taxonomy of Viruses (ICTV) [8].

Lytic Activity of Bacteriophage PP1

Tryptic soy broth (TSB) (Difco) inoculated with an overnight culture of *P. carotovorum* subsp. *carotovorum* Pcc3 was incubated at 28°C with shaking. When the culture reached the early exponential phase (2 × 10^8 CFU/ml), phage PP1 lysate was added at a multiplicity of infection (MOI) of 0.01, 0.1, 1, or 10, respectively, with 10 mM $MgSO_4$. The optical density (600 nm) was measured every 30 min for 24 h (Bioscreen C, Finland). To confirm bacterial viability, phage PP1 lysate was mixed with *P. carotovorum* subsp. *carotovorum* Pcc3 (MOI 5), and viable cells were enumerated at 0, 1, and 3 h post-infection (p.i.). As a negative control, a bacterial culture was inoculated with the same volume of SM buffer instead of phage PP1 lysate.

Stability Towards Temperature and pH

To investigate phage temperature stability, phage aliquots (10^5 PFU/ml) in SM buffer were incubated at 20°C, 30°C, 40°C, 50°C, or 60°C for 1 h. After incubation, viable phage titers were enumerated using a plaque assay. For pH stability, phage aliquots were added to SM buffer (final concentration 10^5 PFU/ml) adjusted with HCl or NaOH to pH 3–12, and incubated at 4°C for 16 h. After incubation, phage viability was determined using a plaque assay.

Host Range Determination

To evaluate the host range of the bacteriophage, *P. carotovorum* subsp. *carotovorum* strains and bacteria of other genera were tested for sensitivity to bacteriophage PP1. *P. carotovorum* subsp. *carotovorum* strains were isolated from various host plants and locations in Korea [28]. The host range of bacteriophage PP1 was assessed using spot tests as published previously with minor modifications [23]. Each bacterial strain was cultured overnight with agitation, and 200 µl aliquots were mixed with 0.4% soft agar containing 10 mM $MgSO_4$. The suspension was then transferred to bottom agar. After solidification, 5 µl of phage lysate (10^{10} PFU/ml) was spotted. The phage sensitivity of bacteria was confirmed by means of the zone of clearance. The bacterial strains used are described in Tables 1 and 2.

Prevention of Soft Rot Disease by Treatment of PP1

Two-week-old lettuces were sprayed with *P. carotovorum* subsp. *carotovorum* Pcc3 (10^8 CFU/ml). After 1 day, a high-titer phage suspension prepared in buffer containing 10 mM $MgCl_2$ was applied to lettuces by spraying. Lettuces were then incubated in closed plastic containers with controlled humidity, and disease progress was monitored for 6 days (recorded every 2 days).

Table 1. Sensitivity of *P. carotovorum* subsp. *carotovorum* isolates to bacteriophage PP1.

Bacteria	Lysis	Bacteria	Lysis	Bacteria	Lysis	Bacteria	Lysis	Bacteria	Lysis
Pcc1	+	Pcc15	+	Pcc29	+	Pcc43	-	Pcc89	-
Pcc2	-	Pcc16	-	Pcc30	-	Pcc44	-	Pcc90	-
Pcc3	+	Pcc17	+	Pcc31	-	Pcc45	-	Pcc91	-
Pcc4	+	Pcc18	-	Pcc32	-	Pcc46	-	Pcc92	-
Pcc5	+	Pcc19	-	Pcc33	-	Pcc47	-	Pcc93	-
Pcc6	-	Pcc20	-	Pcc34	-	Pcc48	-	Pcc94	-
Pcc7	-	Pcc21	+	Pcc35	-	Pcc49	+	Pcc95	-
Pcc8	+	Pcc22	+	Pcc36	-	Pcc50	-	Pcc96	-
Pcc9	+	Pcc23	-	Pcc37	-	Pcc51	-	Pcc97	+
Pcc10	+	Pcc24	-	Pcc38	-	Pcc52	-	Pcc99	+
Pcc11	+	Pcc25	+	Pcc39	-	Pcc56	-	Pcc100	-
Pcc12	+	Pcc26	+	Pcc40	-	Pcc65	-	Pcc101	+
Pcc13	+	Pcc27	+	Pcc41	-	Pcc87	+	Pcc102	+
Pcc14	+	Pcc28	-	Pcc42	-	Pcc88	+	Pcc103	+

Table 2. Bacteria resistant to bacteriophage PP1.

Gram (-) bacteria / *Enterobacteriaceae*		Gram (-) bacteria other than *Enterobacteriaceae*	
Cronobacter sakazakii	ATCC 29544	*Acinetobacter calcoaceticus*	
Dickeya zeae		*Aeromonas hydrophila*	KCTC 2358
Enterobacter cloacae		*Burkholderia andropogonis*	
Escherichia coli O157		*Burkholderia gladioli*	
Klebsiella pneumoniae		*Chryseobacterium balustinum*	
Pantoea agglomerans		*Neisseria meningitidis*	ATCC 13077
Pantoea ananatis	KACC 10059	*Pseudomonas chlororaphis*	SH36
Pectobacterium atrocepticum	KACC 10478	*Pseudomonas corrugata*	SH50
Pectobacterium betavasculorum	KACC 10056	*Pseudomonas fluorescens*	
Pectobacterium chrysanthemi		*Pseudomonas putida*	SH53
Pectobacterium odoriferum	KACC 10486	*Pseudomonas syringae*	
Pectobacterium wasabiae	KACC 10061	*Stenotrophomonas maltophilia*	SHG
Rahnella aquatilis		Gram (+) bacteria	
Salmonella Arizonae	ATCC 13314	*Bacillus cereus*	KCCM 40138
Salmonella Typhimurium	ATCC 13311	*Bacillus megaterium*	
Serratia plymuthica		*Bacillus thuringiensis*	ATCC 29730
Serratia rubidaea		*Clavibacter michiganensis*	KACC 20766
Shigella boydii	ATCC 8700	*Enterococcus faecalis*	ATCC 19433
Shigella flexneri	ATCC 12022	*Listeria innocua*	ATCC 33090
Shigella sonnei	ATCC 25931	*Listeria ivanovii*	ATCC 19119
Yersinia enterocolitica	ATCC 55075	*Listeria monocytogenes*	ATCC 15313
		Staphylococcus epidermidis	KACC 13234
		Staphylococcus pasteuri	KACC 13185
		Staphylococcus xylosus	KACC 10785

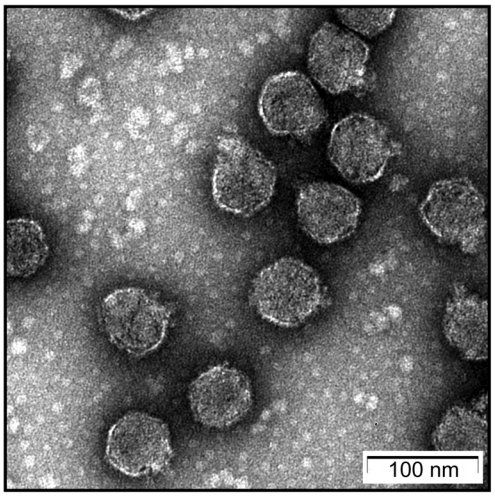

Fig. 1. Transmission electron micrograph of bacteriophage PP1.
The phage was negatively stained with uranyl acetate.

Results and Discussion

Isolation of Bacteriophage PP1

We previously reported the isolation of three types of bacteriophage that infect *P. carotovorum* subsp. *carotovorum* [16]. Of those, one phage isolated from Pyeongchang that generated large plaques with several *P. carotovorum* subsp. *carotovorum* isolates was isolated and designated PP1. Phage PP1 was composed of an icosahedral head with a diameter of ~60 nm and a short, non-contractile tail; thus, it belongs to the *Podoviridae* family of the order *Caudovirales* (Fig. 1). The full genome of bacteriophage PP1 was sequenced [22]. The genome of PP1 was of 44,400 bp with a GC content of 49.74%. A total of 48 open reading frames were predicted and no tRNA genes were detected. The genes encoded by this genome were categorized into five groups; DNA replication/manipulation, phage structure, phage packaging, host lysis, and host specificity. Interestingly, there was no gene involved in lysogen formation, suggesting that bacteriophage PP1 could be an effective biocontrol agent.

Lytic Activity of Bacteriophage PP1 against *P. carotovorum* subsp. *carotovorum*

We assessed the ability of PP1 to lyse host bacteria. A *P. carotovorum* subsp. *carotovorum* culture in the early exponential growth phase was mixed with four PP1 inoculum levels. Although PP1 could effectively lyse its host bacteria, lytic activity differed slightly depending on the MOI (Fig. 2A). At an MOI of 10, bacterial lysis occurred immediately after phage infection. However, the OD_{600} increased slightly from 2.5 h after phage infection, and the rate of increase rose rapidly from 5 h after phage infection. Lysis was delayed according to the PP1 inoculum level used, to 1 (MOI 1), 1.5 (MOI 0.1), or 2 h (MOI 0.01). However, an increased delay in lysis increased the duration of the lysis state. To confirm the lytic activity of bacteriophage, viable bacterial cells were enumerated before and after PP1 treatment (MOI 5) (Fig. 2B). Viable cell numbers were reduced by ~4.2 log (1.6×10^8 to 1.0×10^4 CFU/ml) after 1 h. At 3 h after infection, host cell numbers were increased slightly (6.0×10^4 CFU/ml), but the lysis state was maintained. Thus, PP1 is capable of lysing its host bacteria.

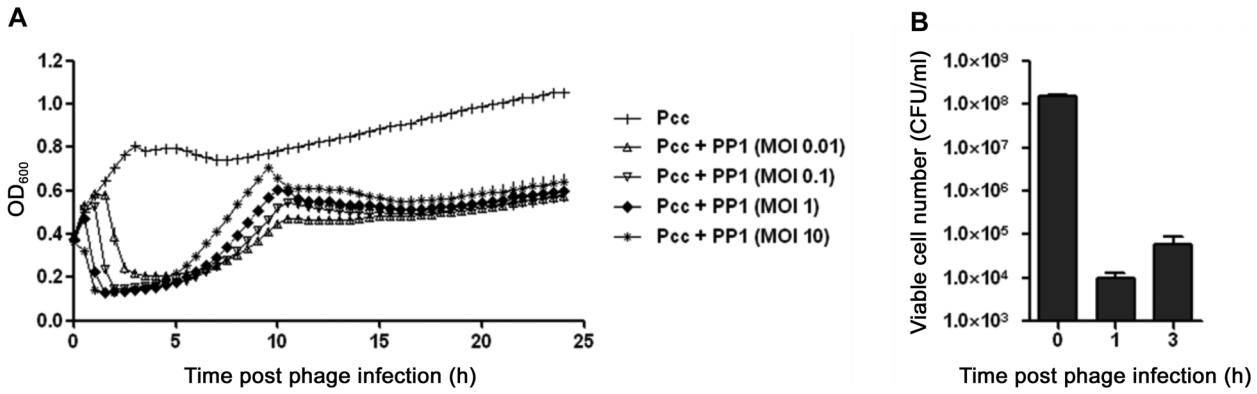

Fig. 2. Lytic activity of bacteriophage PP1 against *P. carotovorum* subsp. *carotovorum*.
(**A**) When the bacterial culture reached the early exponential phase, phage lysate was added at a multiplicity of infection (MOI) of 0.01, 0.1, 1, or 10. The optical density (600 nm) was measured every 30 min for 24 h. (**B**) Viable numbers of *P. carotovorum* subsp. *carotovorum* after PP1 treatment (MOI 5) were compared at 0, 1, or 3 h post-infection.

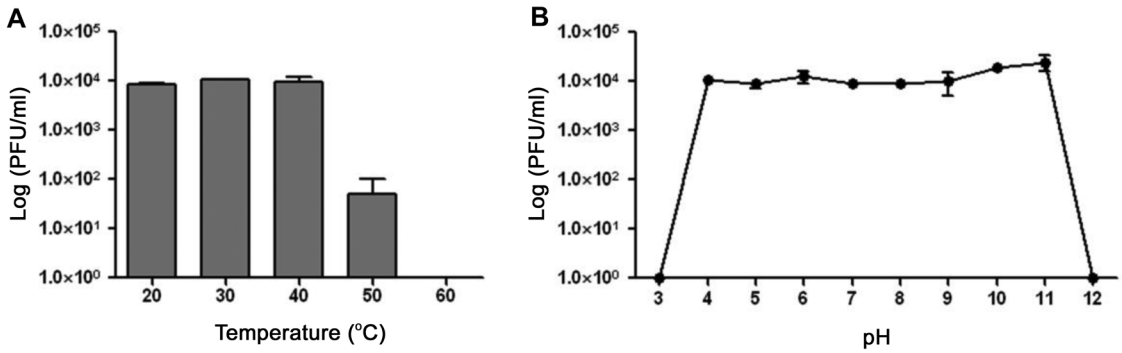

Fig. 3. Stability of bacteriophage PP1 at various temperatures and pH values.
(**A**) PP1 lysate aliquots were incubated at different temperatures for 1 h. (**B**) PP1 lysate aliquot in buffers with various pH values were incubated for 16 h. Next, remnant viable phage titers were determined using plaque assays.

Temperature and pH Stabilities of Bacteriophage PP1

The temperature and pH stabilities of bacteriophage PP1 were evaluated. Phage PP1 was stable at 20–40°C for 1 h (Fig. 3A). However, phage viability was reduced at 50°C and completely inactivated at 60°C. The phage was stable at pH 4–11, but completely inactivated at pH 3 and 12 (Fig. 3B). These results suggest that bacteriophage PP1 was stable at relatively high temperatures and over a wide range of pH values. Under natural field conditions, temperatures do not exceed 50°C; thus, bacteriophage PP1 is sufficiently stable for use as a biocontrol agent in the field.

Antibacterial Activity

To determine the host range of bacteriophage PP1, spot-on-lawn assays on agar plates were performed. A total of 70 *P. carotovorum* subsp. *carotovorum* isolates from the nationwide collection were tested against bacteriophage PP1. We found that 27 *P. carotovorum* subsp. *carotovorum* isolates were susceptible to bacteriophage PP1 (Table 1), but the origin of the isolates was not associated with the susceptibility (data not shown). Both closely related bacteria (*Pectobacterium* sp., *Pantoea*, and *Dickeya*) [1] and bacteria of the same family as *Pectobacterium*, such as *Cronobacter, Enterobacter, Escherichia, Klebsiella, Rahnella, Salmonella, Serratia, Shigella,* and *Yersinia*, were resistant to bacteriophage PP1 [14]. In addition to the *Enterobacteriaceae* strains, other Gram-negative and -positive bacteria were tested. Bacteria of a different order (*Acinetobacter*, *Aeromonas*, *Pseudomonas*, *Stenotrophomonas*), class (*Burkholderia* and *Neisseria*), or even phylum (*Chryseobacterium*) were tested for susceptibility, but none were sensitive to the PP1 bacteriophage (Table 2) [30, 35]. A total of 11 Gram-positive bacteria, representing five genera (*Bacillus*, *Clavibacter*, *Enterococcus*, *Listeria*, *Staphylococcus*), were resistant. Thus, we conclude that bacteriophage PP1 has antimicrobial activity that is relatively specific for *P. carotovorum* subsp. *carotovorum*.

In general, bacteriophage specificity arises by binding to specific receptors. Therefore, differences in the sensitivity to bacteriophages of bacterial strains might be related to variations in their receptors or modification systems [15]. Generally, bacteriophages that can infect a broad range of hosts can potentially be used as biocontrol agents because they are not typically pathogenic. However, their use in phage therapy in animals, humans, or the environment is limited due to the potential for lysis of the resident microbiota [3]. Narrow-host-range bacteriophages (such as PP1) could be applied as a cocktail with other bacteriophages with different receptor specificities. Previous studies explored the usefulness of a phage cocktail [5, 10, 26, 33]. The high target specificity of bacteriophage PP1 is useful, since it is unlikely to infect beneficial bacteria in the phyllosphere or rhizosphere.

Prevention of Soft Rot Disease by PP1

To evaluate the efficacy of bacteriophage PP1 as a biocontrol agent *in planta*, its effect on the development of soft rot disease of lettuce caused by *P. carotovorum* subsp. *carotovorum* was assessed. Lettuces inoculated with *P. carotovorum* subsp. *carotovorum* Pcc3 started to show disease symptoms 2 days p.i. and 80% lettuces showed disease symptoms 6 days p.i. (Fig. 4A). In contrast, PP1-treated lettuces showed a decreased incidence of soft rot disease. The growth of lettuces treated with PP1 after *Pectobacterium* treatment was similar to that of the *Pectobacterium* untreated sample (Figs. 4B, 4C). This indicates that bacteriophage PP1 has biocontrol potential.

In conclusion, bacteriophage PP1 isolated from Pyeongchang is of the *Podoviridae*. Bacteriophage PP1 was small and

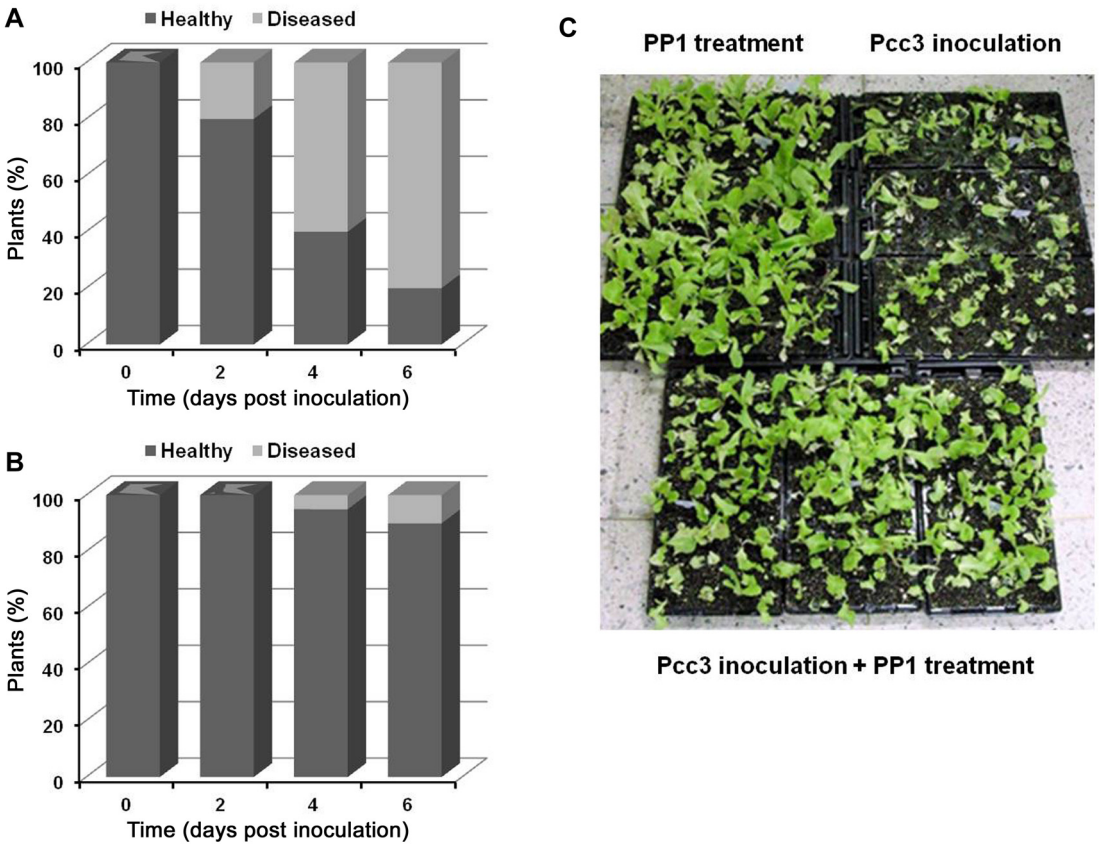

Fig. 4. Prevention of soft rot disease by treatment with bacteriophage PP1.
Two-week-old lettuces were inoculated with *P. carotovorum* subsp. *carotovorum* Pcc3 (day 0). After 24 h (day 1), 10 mM $MgCl_2$ (**A**) or bacteriophage PP1 with 10 mM $MgCl_2$ (**B**) were applied. (**C**) Lettuces observed at 6 days post-infection.

stable at high temperatures and over a broad pH range. Since phage PP1 lysed ~37% of the *P. carotovorum* subsp. *carotovorum* nationwide collection in Korea, it can be used as a cocktail with other *P. carotovorum* subsp. *carotovorum* bacteriophages that have affinities for other receptors. To develop an effective phage cocktail for the control of any type of *P. carotovorum* subsp. *carotovorum* present in Korea, further studies should be done to elucidate the receptors for these bacteriophages.

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