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Evaluation of bacteriophages in the biocontrol of *Pseudomonas syringae* pv. *syringae* isolated from cankers on sweet cherry (*Prunus avium* L.) in Turkey

Mustafa Akbaba^{1*}  and Hatice Ozaktan² 

Abstract

Background: Bacterial canker and subsequent gummosis are caused by multiple pathogens and lead to significant yield and productivity losses in sweet cherry cultivation in Turkey. This study identified that *Pseudomonas syringae* pathovars were responsible for bacterial canker on sweet cherry orchards by using classical and molecular methods and evaluated the biocontrol effects of bacteriophages against *P. syringae* pv. *syringae*.

Results: Pathogenic bacteria were isolated from samples taken from plants showing symptoms of bacterial canker in cherry orchards located in İzmir and Manisa provinces. Specific pathogens were identified using pathogenicity, phenotypic tests, and simplex PCR. Bacteriophages effective against *P. syringae* strains were isolated from soil contaminated with pathogens identified in the diseased orchards using an optimized isolation protocol. The biocontrol activity of bacteriophage isolates against *P. syringae* pv. *syringae* was tested in vitro and in vivo. The results of pathogenicity tests on immature sweet cherry fruits and micropropagated cherry plantlets revealed 10 pathogenic bacteria isolates from 44 plant samples taken from sweet cherry orchards showing symptoms of bacterial canker.

Conclusions: Ten isolates were identified as *Pseudomonas syringae* pv. *syringae*. Nine different pure bacteriophage isolates were effective. The results indicated that bacteriophage isolates may demonstrate variable reactivity against *P. syringae* pathovars.

Keywords: Sweet cherry, Bacterial canker, Bacteriophage, Micropropagation, Plant-associated bacteria

Background

The sweet cherry (*Prunus avium* L.) is believed to have originated in the region that includes the Caspian and Black Sea coasts and subsequently introduced to Europe by birds (Iezzoni et al. 2017). Turkey is the leading global producer of cherries (599,650 tons/year), but they are also propagated on a large scale by the USA, Iran, Chile, Uzbekistan, Italy, and Spain (Faostat 2016). Bacterial canker and subsequent gummosis (hereafter

referred to as bacterial canker) cause significant economic losses to cherry production and yield in all production regions worldwide. Bacterial canker is the general term for diseases caused by multiple disease agents on stone fruit trees (Otto et al. 2017). The most typical symptoms of disease are canker and necrosis, which usually develop around bud eyes, wounds, and branch joints. Because infection is directly related to climatic conditions and pathogens move systemically from the initially infected plant tissues to neighboring tissues, economic and trade-related losses due to bacterial canker may change dramatically from year to year.

* Correspondence: mustafa.akbaba@outlook.com

¹Department of Plant Protection, Faculty of Agriculture, University of Igdir, 76000 Merkez, Igdir, Turkey

Full list of author information is available at the end of the article



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Economic losses result from a reduction in fruit yield and death of branches or entire trees (Ertimurtas 2012).

The study, carried out by Türkoglu et al. (1977), is the first record in Turkey regarding the isolation of *Pss* as the cause of cankers on apricot trees. The causal agents may be harbored on hosts other than cherries, cause systemic infections without symptoms, and develop as epiphytes on non-host plants, making control of the disease challenging (Ertimurtas 2012). Cultural precautions, use of resistant varieties, and treatments with beneficial microorganisms as a biocontrol agent, antibiotics, and copper applications are all used to control bacterial canker (Kennelly et al. 2007), although the use of antibiotics against plant diseases has been prohibited in the European Union since 2007. Bacteriophages are bacteria-specific viruses that infect bacterial cells and use bacterial processes to replicate (Kutter and Sulakvelidze 2004). Bacteriophages have been used effectively as a part of integrated disease management strategies (Buttimer et al. 2017). As it is challenging to definitively diagnose the disease agents that cause bacterial canker in cherry, an integrated approach should be used to identify *P. syringae* isolates by their phenotypic and genomic heterogeneity.

Ten pathogenic bacterial isolates were cultured from cherry orchard samples showing symptoms. Pathogenicity tests are as necessary as biochemical and molecular tests in the detection and diagnostic of *P. syringae* isolates. According to Kałużna and Sobiczewski (2009), tests on immature cherry fruit provided an effective pre-assessment in determining the isolate pathogenicity. The use of micropropagated plantlets to perform bacterial inoculation is a fast and straightforward method of determining disease symptoms in cherry plants without wounding the studied plants or exposing the plants to cold (Vicente and Roberts 2003).

The management of many fruit tree diseases caused by *P. syringae* pathovars is very difficult due to the lack of effective chemical or biological control measures, limited host resistance, and the endophytic nature of the pathogen at some stages of the disease cycle (Kennelly et al. 2007). Fruit trees infected with *P. syringae* pathovars are relatively challenging to treat once disease symptoms begin to occur. The use of bacteriophages rather than standard chemical control measures for plant disease control is a rapidly developing biological control method with great potential in plant protection, especially as antibiotic and copper resistance increases. Therefore, the aim of this study was to identify the species and pathovars responsible for bacterial canker, using classical and molecular methods and utilizing selected bacteriophages (isolated from soils where diseased orchards are planted) against the causal agents of bacterial canker.

Methods

Collection of diseased plant samples

Surveys were conducted during the spring and autumn of 2014 and 2015 in sweet cherry orchards located at 17 different sites in İzmir and Manisa provinces. Samples symptomatic for bacterial canker were randomly collected from different parts of the infected cherry trees (shoots, trunk, leaf, fruit) found in the orchards where bacterial canker was observed. The diseased plant parts were cut from trees using sterile equipment, placed in polyethylene bags, and stored at 4 °C until the isolation process.

Isolation and growth conditions of bacteria

Tissue samples were taken from the leaf, bud, flower, and offshoot showing symptoms of the disease were (surface) sterilized by 70% ethanol (1 min) and sterile water (1 min). The 3-mm fragment was excised in sterile conditions and then crushed in plastic bags containing 10-ml sterile phosphate buffer (0.1 M, pH = 7.2) and incubated in a shaker for 30 min. A 100- μ l aliquot of the well-mixed suspension in plastic bags was inoculated via the spread plate method onto King's B medium (Schaad et al. 2001). Petri dishes were incubated for 24 h at 24 \pm 2 °C. Bacterial colonies that were whitish, round, bright, and vitreous with smooth margins on King's B medium were purified by the streak-plate technique (Cazorla et al. 1998). The 38 purified isolates were stored at -80 °C for long-term use. The production of fluorescent pigment by the bacterial isolates on King's B medium was assessed under 360 nm UV light. Gram reactions were examined using 3% KOH solution. All the bacterial isolates were subjected to a hypersensitivity test using tobacco (Klement et al. 1990; Schaad et al. 2001).

Pathogenicity test on immature fruitlets

Following the method of Kałużna and Sobiczewski (2009), reference strains and all bacterial isolates were subjected to pathogenicity testing in immature cherry fruitlets (PTIF), Cv. "Napoleon" Sweet cherry fruits were allowed to dry after surface sterilization with 70% ethanol (1 min) and sterile water (1 min). Cultures of the bacterial isolates cultivated on King's B medium for 24 h at 24 \pm 2 °C were suspended in sterile water and prepared at a concentration of 10⁹ cfu/ml (OD₆₀₀, 0.1). A 10- μ l droplet of bacterial suspension was pipetted onto each of the marked areas at two inoculation points opened with sterile needles on the disinfected immature sweet cherry fruits. As a positive control, immature sweet cherry fruits were inoculated by *Pss* strain BAY3, *Psm* R1 strain 25B, and *Psm* R2 strain 732 obtained from Poland. The negative control was treated only by sterile distilled water. The treated fruits were incubated in sterile glass Petri dishes (9 mm), on double-layer, moistened

sterile blotting paper at 24 °C and high relative humidity for up to 6 days. At the end of the incubation period, the inoculation points were evaluated as “present” if symptoms were observed or “absent.”

Phenotypic characteristics of bacterial isolates

The pathogenic isolates were further characterized using common phenotypic tests for *P. syringae*. LOPAT tests (levan production [L], oxidase activity [O], pectinolytic activity [P], arginine dihydrolase [A], hypersensitivity to tobacco [T]) were used for each bacterial isolates (Klement et al. 1990; Schaad et al. 2001). Methods commonly used for pathovar discrimination of *P. syringae* strains are GATTa (gelatine hydrolysis [G], aesculin hydrolysis [A], tyrosinase activity [T], tartrate utilization [Ta]) and L-lactate tests (Janse 2010). The *P. syringae* pv. *tomato* (*Pst*) strain DO24, *Pss* strain BAY3 (from our lab collection), *Psm* R1 (race-1) strain 25B, and *Psm* R2 (race-2) strain 732 were used as reference strains. Data published by Janse (2010) (GATTa; *Pss* [++++], *Psm* R1[---+], *Psm* R2 [++--]) were used as diagnostic keys.

Molecular diagnosis of pathogenic bacteria isolates

PCR was used to determine the pathovar and race of *P. syringae* strains isolated from diseased cherry trees. The protocols were generally based on the detection of pathogen toxin coding genes. Molecular diagnosis using PCR was conducted using primer sets (Table 1) and designed for the *syrB* encoding syringomycin production, the *cfl* encoding coronatine toxin production, and the *irp1* encoding yersiniabactin siderophore production (Janse 2010). The results were evaluated using Janse (2010) as a reference for the discrimination of *Pss* (*syrB* [+/-few -], *cfl* [-], *irp1* [-]), *Psm* R1 (*syrB* [-], *cfl* [+/-few -], *irp1* [-]), and *Psm* R2 (*syrB* [-], *cfl* [-], *irp1* [+]).

DNA was extracted from pure culture by the boiling method. All primers were obtained from Medsantek (Turkey). DNA for discrimination of pathovars of *P.*

syringae was amplified using three different PCR primer sets: *cfl* primers (Bereswill et al. 1994), *syrB* primers (Sorensen et al. 1998), and the *irp1* primers (Bultreys et al. 2006). The final reaction mixture for each primer sets was prepared as suggested in the literature (Table 1). Amplification of the bacterial genes was conducted in a thermal cycler (Eppendorf® Mastercycler Personal). The thermal cycling profiles for all primer sets were used (Table 1). PCR products were prepared by 0.5 × TAE buffer (50 × Tris-acetate-EDTA, Fermentas), stained with RedSafeT nucleic acid staining solution (20.000×) (iNtRON Biotechnology), and run on a 1.5% agarose gel at 80 V for 90 min. DNA bands were visualized under UV light using GeneRuler™ 100 bp and 1 kb DNA Ladder (Fermentas).

Pathogenicity of *Pseudomonas syringae* isolates on micropropagated plantlets

For the pathogenicity test, micropropagated cherry plantlets of clone rootstock Ma × Ma 14 (*P. mahaleb* × *P. avium*) (Agromillora, İzmir) were used. Pathogenicity was assessed with isolates that were preliminarily identified as *P. syringae*. Bacterial cultures were grown in King's B medium at 24 ± 2 °C for 24 h, a loopful was used to prepare a suspension in pure, sterile water at a concentration of (10⁹ cfu/ml, OD₆₀₀: 0.1), and 1–2 drops of Tween were added to the suspension. For the inoculation method, a spraying technique was preferred for applying the bacterial isolates to leaves to prevent the creation of wounds on the plants and for ease of application. Micropropagation plantlets (5–6 leaf cherry plants) were replanted into pots 10 cm in diameter containing sterile peat. Approximately 10 ml of the bacterial suspension was sprayed per plantlet in a way that covered the abaxial side of the plant leaves. Pure and sterile water-treated plantlets were evaluated as the negative control, and reference *P. syringae* strains inoculated plantlets as the positive control. After bacterial

Table 1 Primers, amplicon sizes, and references used in the study

Target gene	Primer names	Oligonucleotide sequence	Cycle conditions	Amp. size	References												
irp1	PSYE2	5'-GGCACCTGGAACAGG-3'	The program was as follows: an initial denaturation at 94 °C for 5 min; 37 cycles at 94 °C for 1 min, 64 °C for 1 min, and 72 °C for 1 min; and a final elongation at 72 °C for 6 min	943 bp	Bultreys et al. (2006)												
	PSYE2R	5'-GCCAGATCGTCCATCAT-3'				syrB	Primer B1	5'-CTTCCGTGGTCTTGATGAGC-3'	The program was as follows: an initial denaturation at 94 °C for 5 min; 35 cycles at 94 °C for 1.5 min, 60 °C for 1.5 min, and 72 °C for 3 min; and a final elongation at 72 °C for 10 min	752 bp	Sorensen et al. (1998)	Primer B2	5'-TCGATTTTGC CGTGATGAGTC-3'	cfl	Primer1	5'-GGCGCTCCCTCGCACTT-3'	The program was as follows: an initial denaturation at 93 °C for 2 min; 37 cycles at 93 °C for 1 min, 67 °C for 1 min, and 72 °C for 2 min; and a final elongation at 72 °C for 10 min
syrB	Primer B1	5'-CTTCCGTGGTCTTGATGAGC-3'	The program was as follows: an initial denaturation at 94 °C for 5 min; 35 cycles at 94 °C for 1.5 min, 60 °C for 1.5 min, and 72 °C for 3 min; and a final elongation at 72 °C for 10 min	752 bp	Sorensen et al. (1998)												
	Primer B2	5'-TCGATTTTGC CGTGATGAGTC-3'				cfl	Primer1	5'-GGCGCTCCCTCGCACTT-3'	The program was as follows: an initial denaturation at 93 °C for 2 min; 37 cycles at 93 °C for 1 min, 67 °C for 1 min, and 72 °C for 2 min; and a final elongation at 72 °C for 10 min	650 bp	Bereswill et al. (1994)	Primer2	5'-GGTATTGGCGGGGTGC-3'				
cfl	Primer1	5'-GGCGCTCCCTCGCACTT-3'	The program was as follows: an initial denaturation at 93 °C for 2 min; 37 cycles at 93 °C for 1 min, 67 °C for 1 min, and 72 °C for 2 min; and a final elongation at 72 °C for 10 min	650 bp	Bereswill et al. (1994)												
	Primer2	5'-GGTATTGGCGGGGTGC-3'															

inoculation, the plantlets were stored in polyethylene bags to ensure high humidity, exposed to a 16-h photoperiod at $22 \pm 2^\circ\text{C}$ for 48 h, and then the bags were opened. Trials were conducted in plant growth chamber conditions with four replicates and with one plantlet per replicate. Trials followed a completely randomized design and repeated twice. Symptoms were evaluated 10 days after pathogen inoculation using the 0–9 scale: 0, no symptoms; 1, the presence of small necrotic or chlorotic areas on one leaf of the plant; 3, the presence of necrotic or chlorotic lesions on less than half the plant; 5, the presence of necrotic or chlorotic lesions on half of a plant; 7, the presence of necrotic lesions on more than half of a plant, on the stem and the leaves; and 9, dead plant (Vicente and Roberts 2003). The percent disease index was calculated.

Collection of soil samples for bacteriophage isolation

In 2014 and 2015, soil samples (approximately 30-cm-deep) for isolation of bacteriophages specific for *P. syringae* pathovars were collected from sweet cherry orchards with symptoms of bacterial canker in 17 different locations in İzmir and Manisa provinces. Soil samples containing a small amount of plant debris were collected during spring and autumn seasons from crown projections of cherry trees showing intense bacterial canker symptoms, the samples were then placed in polyethylene bags and stored at 4°C until bacteriophage isolation.

Bacteriophage isolation, purification, and preservation

Isolation of active bacteriophages against *P. syringae* pv. *syringae* was conducted by protocols described in previous studies with appropriate modifications (Crosse and Hingorani 1958; Gašić et al. 2011; Cross et al. 2015). The bacteriophage isolation procedure consisted of the following steps: enrichment, isolation, purification, and preservation.

Enrichment

Liquid enrichment medium (50 ml) was prepared to contain 50 g/l CaCO_3 and 8 g/l nutrient broth in 250-ml conical flasks and sterilized. The cultures of the bacterial isolates developed on the King's B medium for 24 h at $24 \pm 2^\circ\text{C}$ were suspended in sterile distilled water. Flasks were inoculated with 5-ml water suspension with a concentration of 10^9 cfu/ml (OD_{600} : 0.1) of the target bacterial isolate. The flasks were then thoroughly mixed for 1–2 min, and 50 g of sieved moist soil sample was added. The mixture was incubated in the shaker at 160 rpm at $24 \pm 2^\circ\text{C}$ for 24 h.

Isolation

Two different mixtures (each 1 ml) were removed from the soil samples incubated in the shaker at 160 rpm at

$24 \pm 2^\circ\text{C}$ for 24 h. These mixtures were centrifuged in 1.5-ml Eppendorf tubes at 14,000g for 6 min. After centrifugation, approximately 500 μl of the supernatant from each tube was transferred to a new Eppendorf tube. A total of 1000 μl of the supernatant collected from both tubes was mixed, chloroform (10%, v/v) was added, the supernatant was incubated for 20 min, and 500 μl was transferred to a new tube and stored at 4°C for subsequent steps. Spot inoculation and the double agar method were used to observe the formation of phage plaques (Gašić et al. 2011). In both methods, CaCl_2 (1.5 g/l) was added to the medium to enhance the attachment of phages to the host cell surface. The dilution series in the study was prepared within the phage buffer (4 g NaCl, 1.2 g MgSO_4 , 1.2 g Tris-Cl, and 1 L H_2O).

Purification

The phage plaques that appeared to be well-formed were picked from the agar using a sterile toothpick. The toothpick was then, immersed into the Eppendorf tube containing 100 μl of phage buffer, stirred in the buffer, and vortexed slowly. A dilution series was prepared from the phage suspension. After dilution, the double agar and spot inoculation methods were applied. Then, the phage plaques and inhibition zones that appeared by phages were controlled. This phase was repeated three times to ensure the phage isolate was pure.

Preservation

After the final purification phase, phages with well-formed plaques on the double agar medium were allowed to incubate. After plaque formation, 5-ml of phage buffer was added to the Petri dishes. Then, the top agar on the surface was crushed thoroughly. The phage suspension with agar was gently vortexed in Falcon tubes. After centrifugation, approximately 1 ml of supernatant was taken from each tube and slowly filtered through a 0.22- μm filter. The acquired supernatant was treated with 10% (v/v) of chloroform for 20 min. Phages of this final supernatant were stored in the storage medium (NB + 30% glycerol) as triplicates at -80°C . The presence of phage was observed using double-layer agar and spot inoculation methods. Finally, the phage stock was re-tested for vitality control within a few days using the double-layer agar method. After incubation in the medium for 24–48 h, the phage concentration of the -80°C stock was estimated by the bacterial counting formula of Klement et al. (1990) and was considered as plaque-forming units per ml (pfu/ml). Finally, the density that could lyse the bacteria was determined by the spot inoculation method.

In vitro biocontrol activity of bacteriophages against *P. syringae* pathovars

The spot inoculation method was used to check the biocontrol activity of bacteriophages against *P. syringae* pathovars. Briefly, cultures of the bacterial isolates grown on the King's B medium at $24 \pm 2^\circ\text{C}$ for 24 h were prepared at a concentration of 10^9 cfu/ml (OD_{600} : 0.1) in sterile water, then 100 μl of the bacterial suspension was placed onto the center of a sterile Petri dish. Top agar was poured over it, then spread and mixed with the suspension. After medium solidification, 10 μl of the tested bacteriophage suspension was used to spot inoculate the medium surface. According to the inhibition zones appearing after 24–48 h, the results were evaluated as “with effect” or “without any effect.”

In vivo biocontrol activity of bacteriophages against *P. syringae* pathovars

The effects of bacteriophage isolates against *Pss* were determined on micropropagated cherry plantlets in vivo. Phages were revived by dilution and the double-layer agar overlay procedure from phages stocks at -80°C . Phage suspensions added to the medium containing 100 ml of NB-CaCl₂ were incubated on a rotary shaker (160 rpm) for 18–24 h at $24 \pm 2^\circ\text{C}$. They were then centrifuged at 8000g for 5 min, and 2 drops of Tween 80 were added to the resulting phage supernatants. Twelve hours post-inoculation of the pathogen, phage supernatants were sprayed onto micropropagation plantlets at 10 ml per plantlet. Phages were applied to the plantlets individually and in combination (5 phage isolates) (Boulé et al. 2011). Positive control plantlets were treated with NB-CaCl₂, which was incubated on a rotary shaker for 18–24 h and also *Pss* suspension. Negative control plantlets were treated with NB-CaCl₂ and incubated on a rotary shaker for 18–24 h, and sterile water. Twelve hours before the phage treatment, approximately 10-ml pathogenic bacteria was sprayed onto each micropropagation cherry plantlet. Treated plantlets were kept in the darkness for 12 h to increase the bacteriophages' chance to adapt and survive against UV radiation. The treated plantlets were kept in polyethylene bags to ensure high humidity (approximately 96%) in a 16-h photoperiod at $22 \pm 2^\circ\text{C}$ for 48 h, and then, the bags were opened. Simultaneously, the phage density in each phage supernatant treatment (1 ml) was calculated by the double-layer agar. Trials were conducted in plant growth chamber conditions with four replicates and with one plantlet (5–6 leaf plant) per replicate. Trials were designed according to a completely randomized and repeated twice. Symptoms were evaluated, 10 days after pathogen inoculation using the 0–9 scale, and disease severity values were obtained (Vicente and Roberts 2003). The percentage disease index was calculated.

Statistical analysis

All data were analyzed using Microsoft Excel 2010 and SPSS (IBM SPSS Statistics, version 18.0). Variance analysis (ANOVA) was applied, and the differences among the averages were determined by Duncan's multiple comparison test. The percent of the disease index was calculated using the formula $\text{DI} = (\Sigma [\text{Number of plants in the rating} \times \text{Rating number}] / [\text{total number of plants} \times \text{the highest rating}]) \times 100\%$ (Liu et al. (1995)).

Results

Establishment of a bacterial collection

Thirty-eight bacterial isolates were isolated from 44 plant samples showing disease symptoms. Bacterial colonies could not be isolated due to excessive fungal growth in 6 out of 44 diseased plant samples. From the rest of the samples, 38 candidate bacteria potentially pathogenic on sweet cherry were isolated with characteristic colony morphology. However, most of the bacterial isolates were determined not to be the causal agents of bacterial canker by the results of the pathogenicity and other tests. These bacterial isolates were considered to be non-pathogenic or saprophytic species that survived due to symptoms/disorders caused by other pathogens and abiotic factors. Among the 38 isolates, 10 isolates were selected as they met the following criteria: Gram (-), HR in tobacco (+), and pathogenicity test in immature cherry fruit (+). These isolates were identified as BY3L238, A2L245, Ö3L2308, A3F1310, K2L312, Ö3L1315, BY5L316, AL1T3324, AL1T1343, and HAL3T348.

Phenotypic characteristics of bacterial isolates

Ten isolates among the 38 isolates were determined to be pathogenic according to the results of the pathogenicity test on immature fruits. Then, they were subjected to LOPAT and GATTa tests. According to these test results, 10 isolates matched with reference *Pss* strain BAY3 isolate, and they were different from other reference strains (Table 2).

Molecular detection of bacterial isolates

According to the primer set designed for the *syrB* gene used by Sorensen et al. (1998) and the PCR results, 10 isolates together with *Pss* strain BAY3 as a reference showed the presence of a 752-bp fragment (references: *Psm* R1 strain 25B [-], *Psm* R2 strain 732 [-]). According to the primer set designed for the *cfl* used by (Bereswill et al. 1994) and the PCR results, *P. syringae* pv. *tomato* [*Pst*] strain DO24, which is used for positive control purposes and known to produce the coronatin toxin, showed the amplification of a 650-bp fragment. Ten isolates and the reference strains *Pss* BAY3, *Psm* R1 strain 25B, and *Psm* R2 strain 732 did not show the presence

Table 2 Pathogenicity results on immature fruits (PTIF), LOPAT, GATTa, and PCR (syrB, cfl, irp1) results of bacterial isolates

Isolate code	Phenotypic tests													PCR		
	PTIF	Gr	Fp	Le	Ox	Pe	Ar	T	Ge	Ae	Ty	Ta	La	syrB	cfl*	irp1
<i>Pss</i> BAY3	+	-	+	+	-	-	-	+	+	+	-	-	+	+	-	-
<i>Psm</i> 25B	+	-	+	+	-	-	-	+	-	-	+	+	-	-	-	-
<i>Psm</i> 732	+	-	+	+	-	-	-	+	+	-	-	-	-	-	-	+
BY3L238	+	-	+	+	-	-	-	+	+	+	-	-	+	+	-	-
A2L245	+	-	+	+	-	-	-	+	+	+	-	-	+	+	-	-
Ö3L2308	+	-	+	+	-	-	-	+	+	+	-	-	+	+	-	-
A3F1310	+	-	+	+	-	-	-	+	+	+	-	-	+	+	-	-
K2L312	+	-	+	+	-	-	-	+	+	+	-	-	+	+	-	-
Ö3L1315	+	-	+	+	-	-	-	+	+	+	-	-	+	+	-	-
BY5L316	+	-	+	+	-	-	-	+	+	+	-	-	+	+	-	-
AL1T3324	+	-	+	+	-	-	-	+	+	+	-	-	+	+	-	-
AL1T1343	+	-	+	+	-	-	-	+	+	+	-	-	+	+	-	-
HAL3T348	+	-	+	+	-	-	-	+	+	+	-	-	+	+	-	-

Gr gram reaction, Fp fluorescent pigment production, Le levan formation, Ox oxidase activity, Pe pectinolytic activity, Ar arginine dihydrolase, T (Hr) hypersensitivity of tobacco, Ge gelatine hydrolysis, Ae aesculin hydrolysis, Ty tyrosinase activity, Ta utilization of tartaric acid, La utilization of lactate

**P. syringae* pv. *tomato* (*Pst*) strain DO24 was used as a positive control at the amplification of the *Cfl* gene

of a 650-bp fragment. According to the primer set designed for the *irp1* used by Bultreys et al. (2006) and PCR results, *Psm* R2 strain 732 as a reference of race-2 was the only isolate that showed amplification of a 943-bp fragment (Table 2), and that was the only fragment of interest found in that isolate. Ten of the isolates from the study (BY3L238, A2L245, Ö3L2308, A3F1310, K2L312, Ö3L1315, BY5L316, AL1T3324, AL1T1343, HAL3T348) were verified as *Pss* according to the PCR results for 3 different genes, phenotypic characteristics, pathogenicity, and molecular diagnostics.

Pathogenicity of *Pseudomonas syringae* isolates on micropropagated plantlets

The average results of two trials of pathogenicity assays performed on 10 *P. syringae* isolates and 3 reference strains (*Pss/Psm* R1/*Psm* R2) are presented (Table 3). The average disease index was 37.1% in the positive control plantlets inoculated with reference *Pss* strain BAY3, and a higher disease index was reported in Ö3L2308, BY5L316, the coded *Pss* isolates, and the reference *Psm* R2 strain 732 isolate compared to the positive control. In cherry plantlets, the pathogenicity of *Pss* isolate BY5L316 was found to be statistically significant, with a 46.5% disease index rate compared to the reference *Pss* strain BAY3 and all other isolates.

Isolation, purification, and preservation of bacteriophages

Soil samples were taken from 17 different locations in İzmir and Manisa provinces in 2014, but no bacteriophage could be isolated due to the unsuitable sampling time (the initial incidence of the bacterial infection). In

2015, the collection of 36 different soil samples allowed for successful isolation of nine different pure phage isolates (Φ911, Φ922, Φ943, Φ954, Φ1215, Φ1226, Φ137, Φ358, and Φ369) from soil samples collected from four locations (Ören, Bağyurdu, Yukarıcobanisa, and Aşağıcobanisa). Bacteriophage isolates purified during the last

Table 3 Pathogenicity results of *Pseudomonas syringae* isolates on micropropagated cherry plantlets within a growth chamber

Average of experiments I-II	
Isolate code	Average disease index (%)*
K2L312	16.7 ± 0.86 a**
BY3L238	18.5 ± 0.51 a-c
HAL3T348	20.4 ± 0.93 a-b
AL1T1343	25.9 ± 0.51 a-d
A2L245	29.6 ± 0.40 a-d
Ö3L1315	29.6 ± 0.40 a-d
A3F1310	29.6 ± 0.65 a-d
<i>Pss</i> BAY3	37.0 ± 0.61 b-d
AL1T3324	37.0 ± 0.61 b-d
<i>Psm</i> 25B	37.0 ± 0.82 b-d
Ö3L2308	40.7 ± 0.35 cd
<i>Psm</i> 732	40.7 ± 0.75 b-d
BY5L316***	46.5 ± 0.43 d

*The results are an average of the two trials. Four plantlets were evaluated in each trial for each treatment

**According to Duncan's multiple comparison test, the difference between the averages indicated by the same letters in the same column is insignificant according to $P < 0.05$

***The most virulent isolate, BY5L316, was selected to use in the next stage of the study

stage were aliquoted into cryoprotectant tubes containing glycerol and NB liquid medium for long-term preservation and stored at -80°C . The phage density (pfu/ml) in these tubes was calculated according to Klement et al. (1990) (Table 4). For control purposes, the phages were proven to be present in the tubes by reviving studies from the stock after 48 h. Five different phage isolates (Φ 1215, Φ 1226, Φ 137, Φ 358, Φ 369) with high lytic activity against *Pss* were selected for future studies.

In vitro biocontrol activity of bacteriophages against *P. syringae* pathovars

Bacteriophages against different bacterial isolates showed a range of biocontrol activities (Fig. 1a, Table 5). *Pss* BAY3 was lysed highly by all phage isolates (Fig. 1b). *Psm* R1 strain 25B was lysed very effectively by phage isolates Φ 1215 and Φ 137 (Fig. 1c). *Psm* R2 strain 732 was lysed very effectively by only phage isolate Φ 369 (Fig. 1d). In contrast to the other *Pss* isolates, the *Pss* HAL3T348 isolate was unaffected by the other phage isolates except for Φ 358 and Φ 369.

In vivo biocontrol activity of bacteriophages against *P. syringae* pathovars

In trials conducted on micropropagated cherry plantlets in the growth chamber, five bacteriophage isolates and their combinations were used to determine the biocontrol activities against the high-virulence *Pss* strain BY5L316. The density of bacteriophages in the treatments was reported in Table 6. Average results of two separate trials demonstrated that at 10 days post-inoculation, 4 out of 6 phage treatments of the phage treatments (Φ 1226, Φ 137, Φ 358 [Fig. 2a], Φ 369) reduced successfully more than 50% of the disease incidence caused by *Pss* strain BY5L316 compared to the control (48.1%, Fig. 2b). Except for Φ 1215 treatment, there was a statistically significant difference between controls and all phage treatments (Table 6).

Combination phage treatment decreased the disease incidence by 42.3%, less than individual phages.

Discussion

The use of phages for the diagnosis of causal agents of bacterial diseases was introduced in Turkey when it was discovered that bacteriophages could attach to the receptors on the cell wall of bacteria (Erkan and Saygılı 1987). This study is one of the first attempts at the use of bacteriophages in the biological control of plant bacterial diseases in Turkey. In recent years, products containing bacteriophages against plant bacterial diseases have been launched commercially by companies based in the USA, Hungary, and Scotland (Buttimer et al. 2017). Bacteriophages with specific effects against *Psm* NA3 of the causal agents of bacterial canker were isolated first from cherry, plum, and apricot orchards across England (Crosse and Hingorani 1958). The phages were isolated from soil against *P. syringae* pv. *syringae* which were a causal agent of bacterial canker on sweet cherry in this study. One of the most critical aspects of phage isolation from the soil was sampling time. Payne and Jansen (2001) emphasized that phage treatment must be carefully timed to coincide with a sufficiently dense bacterial population in order to be effective phage therapy. With the emergence of bacterial canker symptoms and the progression of infection, the source of the bacteriophages in the soil was thought to be diseased plant residuals and the products of gummosis, which was likely why bacteriophages have not been isolated from the soil samples collected when the symptoms of bacterial canker firstly appear. Bacteriophages were only found at high titers in soil under conditions suitable for the proliferation of their hosts (Reaney and Marsh 1973). At the phage-host system, it was reported that susceptible host cell populations must reach a critical density before phage replication can occur. In the same study, it has been reported that there was a longer delay before phage numbers increase over the numbers

Table 4 List of bacteriophages isolated from soil

No.	Isolate code	Sample location	pfu/ml (-80 stock)	Plaque sizes (\sim mm)	Plaque forms on solid media
1	Φ 911	Ören-6	9×10^7	1.15	Smallest and turbid
2	Φ 922	Ören-6	2×10^6	0.98	Smallest and clear
3	Φ 943	Ören-6	5×10^8	2.62	Medium and turbid
4	Φ 954	Ören-6	2×10^6	2.48	Medium and turbid
5	Φ 1215	Bagyurdu-7	3×10^8	4.37	Largest and clear
6	Φ 1226	Bagyurdu-7	5×10^8	4.50	Largest and clear
7	Φ 137	Ören-5	6×10^8	4.22	Largest and clear
8	Φ 358	Y. Çobanisa-2	2×10^9	1.00	Smallest and clear
9	Φ 369	A. Çobanisa-3	8×10^9	0.95	Smallest and turbid

Plaques of different diameter (Φ) are shown: $\Phi < 2$ mm (smallest), $\Phi = 2-4$ mm (medium), and $4 \text{ mm} < \Phi$ (largest)

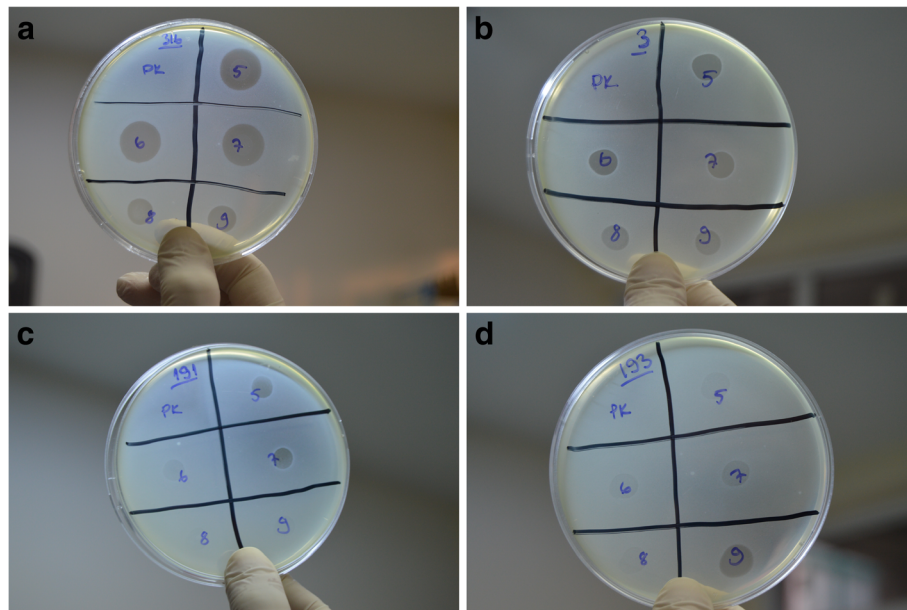


Fig. 1 Lytic effects of bacteriophages (Φ1215 [5]; Φ1226 [6]; Φ137 [7]; Φ358 [8]; Φ369 [9]) against *Pseudomonas syringae* pathovars. **a** *Pss* strain BY5L316 (the most virulent pathogenic isolate). **b** *Pss* strain BAY3 [3]. **c** *Psm* R1 strain 25B [191]. **d** *Psm* R2 strain 732 [193]). *Numbers in brackets show the location of strains/phages in lab strain/phage collection (e.g., 3 instead of *Pss* strain BAY3 or 5 instead of Φ1215)

of input phage when host cell densities were very low (Kasman et al. 2002). Pelzek et al. (2013) reported that CaCl₂ improved phage adsorption during the infection process of the host bacteria. It was proposed that to increase the possibility of isolating phages specific to bacteria, the concentrations of CaCO₃ in the isolation protocol and CaCl₂ in the double-layer method were important, as they affected the success of phage isolation from the soil by allowing the bacteriophages to attach to the bacterial surface. The protocol used for the isolation of phages from soil against causal agents of bacterial canker has been optimized with information collected from different studies and has been used successfully in this study.

The reactions of five phages isolated against three pathogenic *Pss* agents were detected in vitro. All phages isolated showed substantial lytic effect against *Pss*. In addition, different reactions to bacteriophages were observed among *Pss* strains. Bacteriophage isolates Φ1215, Φ1226, and Φ137 showed a lytic effect on all *Pss* strains except HAL3T348. A widespread belief has emerged, particularly in the field of medicine, that considers the broad-spectrum activity of antibiotics and the narrower spectrum of phages; the failure of phages could be much higher if the pathogen to which the phages will be applied but not identified correctly (Kutter and Sulakvelidze 2004). It was also important because phage isolates that were isolated for one pathovar may have different

Table 5 Host range data between some pathogenic *Pseudomonas syringae* isolates and bacteriophage isolates

Phages ^c	Pathogen isolates ^a											AL1T3324	Pss AL1T1343 HAL3T348
	Pss ^b BAY3	Psm 25B	Psm 732	Pss BY3L238	Pss A2L245	Pss Ö3L2308	Pss A3F1310	Pss K2L312	Pss Ö3L1315	Pss BY5L316	Pss		
Φ1215	+	+	*	+	+	+	+	+	+	+	+	+	-
Φ1226	+	*	*	+	+	+	+	+	+	+	+	+	-
Φ137	+	+	*	+	+	+	+	+	+	+	+	+	-
Φ358	+	*	*	+	+	+	+	+	+	+	+	+	+
Φ369	+	*	+	+	+	+	+	+	+	+	+	+	+

^a*Pseudomonas syringae* pv. *syringae* (*Pss*), *P. syringae* pv. *morsprunorum* [*Psm*] race 1 (*Psm* 25B), *P. syringae* pv. *morsprunorum* [*Psm*] race 2 (*Psm* 732)

^b(+) lysis after infection with tested bacteriophage, (-) a lack of lysis after infection with tested bacteriophage, (*) a weak of lysis after infection with tested bacteriophage

^cUndiluted phage stocks in Table 4 were used for this test

Table 6 Biocontrol (%) at bacteriophage treatments on *Pseudomonas syringae* pv. *syringae* disease's severity in micropropagated cherry plantlets within a growth chamber

Treatments	Treatment concentration (pfu/ml)	Average disease index (%) ^a	Efficacy (%) ^b
Φ1226	$2,2 \times 10^9$	9.7 ± 0.75 a ^c	79.8
Φ137	$1,5 \times 10^9$	13.9 ± 0.77 ab	71.2
Φ358	$4,1 \times 10^{10}$	15.3 ± 0.63 ab	68.3
Φ369	6×10^{10}	20.8 ± 1.10 ab	56.7
ΦCombination of phages	5×10^9	27.8 ± 0.54 b	42.3
Φ1215	4×10^9	38.9 ± 0.49 c	19.2
BY5L316 (control +)	0	48.1 ± 0.51 c	0.0

a. The results are an average of the two trials. Four plantlets were evaluated in each trial for each treatment

b. Percentage reduction in diseases severity compared to plants treated with pathogen alone

c. Means within columns sharing a letter in common are not significantly different ($P < 0.05$; Duncan's test). The standard error of each set is displayed

levels of effectiveness for different pathovars of the same species.

It was also found that 66% of the phage treatments reduced successfully more than 50% of the disease incidence caused by *Pss* strain BY5L316 than the control. In a similar study, bacteriophages have been used alone and in combination with copper bactericides to control the Asiatic citrus canker and citrus bacterial spot caused by *Xanthomonas axonopodis* pathovars *citri* and *citrumelo*, respectively. Although phage treatment was less effective than copper-mancozeb in greenhouse trials, it has significantly reduced the disease severity of citrus canker. Combinations of phage and copper-mancozeb treatments did not show good results compared to copper-mancozeb treatment alone against citrus canker. In the same study, while phage treatment did not reduce the severity of disease of citrus bacterial spot on susceptible grapefruits, it reduced disease severity significantly on the moderately susceptible Valencia oranges in trials. Phage treatment provided a moderate level of control over the citrus bacterial spot in a commercial nursery

contaminated with diverse populations of the pathogen (Balogh et al. 2008).

The fact that bacteriophages have effects against multiple disease agents has advantages and disadvantages in terms of biological control of bacterial disease agents. The specificity of bacteriophages to the host and the range of pathogens it affects are critical in combatting bacterial canker caused by multiple bacterial disease agents. In this study, the lytic effect of some isolated bacteriophages against different agents was observed. The phage combinations in different proportions may increase the success of the control against bacterial canker. In terms of biocontrol, the probability of finding a bacterium to be adsorbed by a single phage depended on the time, the density of the phage, the phage diffusion rate, and the local density of the bacteria sensitive to the phage. The more bacteria present will result in faster phage adsorption (Kasman et al. 2002). However, when preparing these combinations, particular attention should be paid to the receptors recognized by a particular phage on a bacterial target. It has been reported that

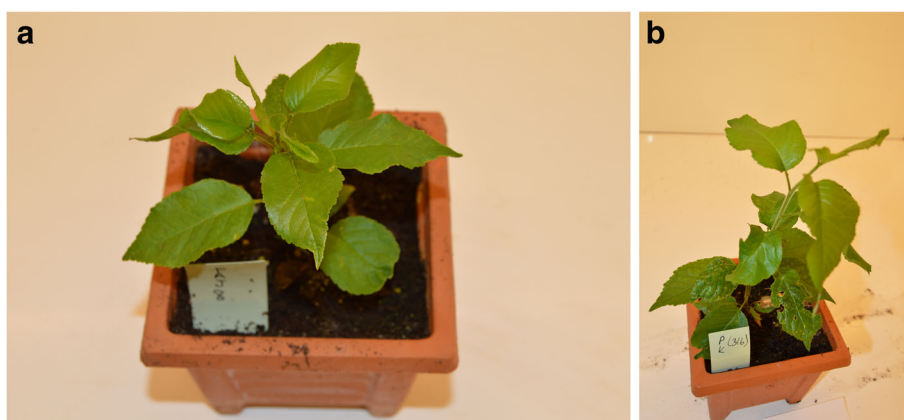


Fig. 2 a Plantlet treated with Φ358 [8] phage treatment on 10 days post-inoculation with *Pss* strain BY5L316. **b** Control (+) plantlet treated with only *Pss* strain BY5L316 on 10 days post-inoculation

generating phage mixtures may be helpful where the probability of host resistance was low (Frampton et al. 2012) and may lead to the development of phage combinations in which individual members work in synergy to eliminate the target bacteria (Born et al. 2011). In addition to these advantages, the bacteriophages having a wide host range, the possibility of bacteriophage's ineffectiveness, or the possibility of reducing the product yield by causing the death of non-target beneficial and antagonistic bacteria were among the disadvantages of bacteriophage application (Ahmad and Morgan 1994). In a similar study, samples were collected from 6 different geographical sites in England. A total of 70 phages were isolated from soil (60), leaves (1), and bark (9) of cherry trees. The host specificity of the phage isolates was assessed against strains of pathovars of *P. syringae*. Host-range experiments showed that it can infect/lyse not only different isolates of *Pss* and *Psm* but also other *P. syringae* pathovars, including *P. s. pv. tomato* DC3000, *P. s. pv. lachrymans* 789, and *P. cichorii*. However, the phages were unable to infect beneficial bacteria such as *P. fluorescens*. Two phage cocktails and 13 individual phage treatments were used against cultures of *Pss*, *Psm1*, and *Psm2* to generate killing curves. All treatments were found effective for reducing the density of the bacterial host. However, there were substantial differences in the dynamics of bacterial killing by different groups of phages. Various concentrations of phages demonstrated different effects on bacterial populations. The highest titer of phage, the greatest their ability to inhibit the growth of the bacterial host, and in less time. A key consideration for phage use in biocontrol was storage and efficacy after storage. It has been reported that the largest the phage plaque, the best the phage survival at different temperatures (Rabiey et al. 2020).

In contrast, the combination of phages was less successful than specific individual phage treatments in this study. This result may be due to the fact that the ratio between phage and host bacterial density was not at the desired level or that phages applied in combination might compete among themselves. A related study emphasized the importance of multiple infections for natural phage populations. When a host was infected with multiple phages, it was shown that the lytic activity of prophages declined. The competition for the host among phages has been shown to be variable for each individual phage. Exploitation competition has been bound to occur, limiting resources in a bacterial cell. It has been suggested that interaction between two simultaneously executed lytic programs within the same cell may hinder efficient resource depletion. It has been reported that phages can be ranked according to their within-host competitive abilities. A phage that dominated intracellular processes was competitively superior (Refardt 2011).

In support of obtained results, a previous study found that one undesired feature of some phages was preventing the host's superinfection by the same or a closely related phage during phases of infection. Some phages in the same study were reported to prevent secondary infection of the host by another phage (Lu and Henning 1994).

Conclusions

Pss was found to be the most common disease agent of bacterial canker on cherry orchards in İzmir and Manisa provinces, Turkey. In vivo and in vitro results demonstrated that bacteriophage treatments had biocontrol effects against *Pss* via reducing the severity of the disease. In further studies, isolated bacteriophage particles will be observed using transmission electron microscopy (TEM) and investigate effective and protective formulations of bacteriophages.

Abbreviations

Pss: *P. syringae* pv. *syringae*; *Psm*: *P. s. pv. morsprunorum*; *Pst*: *P. syringae* pv. *tomato*; PTIF: Pathogenicity testing in immature fruits; pfu: Plaque-forming unit; cfu: Colony-forming unit

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Authors' contributions

This article was produced from the corresponding author's thesis of the doctorate. MA contributed to the different stages of the process of the research such as research concept and design, collection, and/or assembly of the data; data analysis and interpretation; and writing the article (contribution rate 60%). HO conceived the study and participated in its design and coordination (contribution rate 40%). Both authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests. All authors have reviewed the manuscript and approved its submission to the *Egyptian Journal of Biological Pest Control*.

Author details

¹Department of Plant Protection, Faculty of Agriculture, University of Igdir, 76000 Merkez, Igdir, Turkey. ²Department of Plant Protection, Faculty of Agriculture, University of Ege, 35100 Izmir, Turkey.

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References

- Ahmad M, Morgan V (1994) Characterization of a cowpea (*Vigna unguiculata*) rhizobiophage and its effect on cowpea nodulation and growth. *Biol Fertl Soils* 18:297–301. <https://doi.org/10.1007/BF00570632>
- Balogh B, Canteros BI, Stall RE, Jones JB (2008) Control of citrus canker and citrus bacterial spot with bacteriophages. *Plant Dis* 92(7):1048–1052
- Bereswill S, Bugert P, Völksch B, Ullrich M, Bender CL, Geider K (1994) Identification and relatedness of coronatine-producing *Pseudomonas syringae* pathovars by PCR analysis and sequence determination of the amplification products. *Appl Environ Microbiol* 60:2924–2930
- Born Y, Fieseler L, Marazzi J, Lurz R, Duffy B, Loessner MJ (2011) Novel virulent and broad-host-range *Erwinia amylovora* bacteriophages reveal a high degree of mosaicism and a relationship to Enterobacteriaceae phages. *Appl Environ Microbiol* 77(17):5945–5954. <https://doi.org/10.1128/AEM.03022-10>
- Boulé J, Sholberg PL, Lehman SM, O'gorman DT, Svircev AM (2011) Isolation and characterization of eight bacteriophages infecting *Erwinia amylovora* and their potential as biological control agents in British Columbia. *Can J Plant Pathol* 33(3):308–317. <https://doi.org/10.1080/07060661.2011.588250>
- Bultreys A, Gheysen I, de Hoffmann E (2006) Yersiniabactin production by *Pseudomonas syringae* and *Escherichia coli*, and description of a second yersiniabactin locus evolutionary group. *Appl Environ Microbiol* 72:3814–3825. <https://doi.org/10.1128/AEM.00119-06>
- Buttimer C, McAuliffe O, Ross RP, Hill C, O'Mahony J, Coffey A (2017) Bacteriophages and bacterial plant diseases. *Front Microbiol* 8:34. <https://doi.org/10.3389/fmicb.2017.00034>
- Cazorla FM, Torés JA, Olalla L, Pérez-García A, Farré JM, de Vicente A (1998) Bacterial apical necrosis of mango in southern Spain: a disease caused by *Pseudomonas syringae* pv. *syringae*. *Phytopathology* 88:614–620. <https://doi.org/10.1094/PHYTO.1998.88.7.614>
- Cross T, Schoff C, Chudoff D et al (2015) An optimized enrichment technique for the isolation of *Arthrobacter* bacteriophage species from soil sample isolates. *J Vis Exp*. <https://doi.org/10.3791/52781>
- Crosse J, Hingorani M (1958) A method for isolating *Pseudomonas mors-prunorum* phages from the soil. *Nature* 181:60–61. <https://doi.org/10.1038/181060a0>
- Erkan S, Saygılı H (1987) The preliminary studies on the isolation of the bacteriophages of some phytopathogenic bacteria. *J Turki Phytopathol* 16: 71–76
- Ertimurtas D (2012) Sert çekirdeklilerde bakteriyel kansere neden olan *Pseudomonas syringae* pathovarlarının klasik ve moleküler yöntemlerle tanisi. Yüksek Lisans Tezi (MSc), Ege Üniversitesi Fen Bilimleri Enstitüsü <https://tez.yok.gov.tr/UlusalTezMerkezi/tezSorguSonucYeni.jsp>. Accessed 07 Jan 2021
- Faostat (2016) Production of cherries: top 10 producers. <http://www.fao.org/faostat/en/#data/QC/visualize>. Accessed 18 Jan 2018.
- Frampton RA, Pitman AR, Fineran PC (2012) Advances in bacteriophage-mediated control of plant pathogens. *Int J Microbiol* 2012:326452. <https://doi.org/10.1155/2012/326452>
- Gašić K, Ivanović MM, Ignjatov M, Čalić A, Obradović A (2011) Isolation and characterization of *Xanthomonas euvesicatoria* bacteriophages. *J Plant Pathol* 93(2):415–423
- Iezzoni A, Wünsch A, Höfer M et al (2017) Biodiversity, germplasm resources and breeding methods. In: Cherries: botany, production and uses. CABI International, pp 36–59. <https://doi.org/10.1079/9781780648378.0036>
- Janse J (2010) Diagnostic methods for phytopathogenic bacteria of stone fruits and nuts in COST 873. *EPPO Bulletin/Bulletin OEPP* 40:68–85
- Kałużna M, Sobiczewski P (2009) Virulence of *Pseudomonas syringae* pathovars and races originating from stone fruit trees. *Phytopathologia* 54:71–79
- Kasman LM, Kasman A, Westwater C, Dolan J, Schmidt MG, Norris JS (2002) Overcoming the phage replication threshold: a mathematical model with implications for phage therapy. *J Virol* 76:5557–5564. <https://doi.org/10.1128/jvi.76.11.5557-5564.2002>
- Kennelly MM, Cazorla FM, de Vicente A, Ramos C, Sundin GW (2007) *Pseudomonas syringae* diseases of fruit trees: progress toward understanding and control. *Plant Dis* 91:4–17. <https://doi.org/10.1094/PD-91-0004>
- Klement Z, Rudolph K, Sands D (1990) Methods in phyto bacteriology. Akademiai Kiado, Budapest, XIV, p 568
- Kutter E, Sulakvelidze A (2004) Bacteriophages: biology and applications. CRC Press, Boca Raton, Florida, pp 5–27
- Liu L, Kloepper J, Tuzun S (1995) Induction of systemic resistance in cucumber against *Fusarium* wilt by plant growth-promoting rhizobacteria. *Phytopathology* 85:695–698
- Lu MJ, Henning U (1994) Superinfection exclusion by t-even-type coliphages. *Trends Microbiol* 2(4):137–139
- Otto M, Petersen Y, Roux J, Wright J, Coutinho TA (2017) Bacterial canker of cherry trees, *Prunus avium*, in South Africa. *Eur J Plant Pathol* 151:427–438. <https://doi.org/10.1007/s10658-017-1384-5>
- Payne RJ, Jansen VA (2001) Understanding bacteriophage therapy as a density-dependent kinetic process. *J Theor Biol* 208(1):37–48
- Pelzek AJ, Schuch R, Schmitz JE, Fischetti VA (2013) Isolation, culture, and characterization of bacteriophages. *Current Protocols* 7(1):4.4.1–4.4.33. <https://doi.org/10.1002/9780470089941.et0404s07>
- Rabiey M, Roy SR, Holtappels D, Franceschetti L, Quilty BJ, Creeth R et al (2020) Phage biocontrol to combat *Pseudomonas syringae* pathogens causing disease in cherry. *Microb Biotechnol* 13(5):1428–1445. <https://doi.org/10.1111/1751-7915.13585>
- Reaney DC, Marsh SCN (1973) The ecology of viruses attacking *Bacillus stearothermophilus* in soil. *Soil Biol Biochem* 5(4):399–408
- Refardt D (2011) Within-host competition determines reproductive success of temperate bacteriophages. *ISME J* 5(9):1451–1460. <https://doi.org/10.1038/ismej.2011.30>
- Schaad NW, Jones JB, Chun W (2001) Laboratory guide for the identification of plant pathogenic bacteria. vol ed. 3. American Phytopathological Society (APS Press), St Paul, USA, p 373
- Sorensen KN, Kim KH, Takemoto JY (1998) PCR detection of cyclic lipodepsinonapeptide-producing *Pseudomonas syringae* pv. *syringae* and similarity of strains. *Appl Environ Microbiol* 64:226–230
- Türkoglu K, Cınar Ö, Öktem Y (1977) Sivas ve Malatya illerinde kayısı ağaçlarında kurumaların sebepleri ve en uygun mücadele metodunun tesbiti üzerinde araştırmalar. TÜBİTAK Yayınları, p 332
- Vicente J, Roberts S (2003) Screening wild cherry micropropagated plantlets for resistance to bacterial canker. In: *Pseudomonas syringae* and related pathogens. Springer, pp 467–474

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