



Research article

Broad host range bacteriophages found in rhizosphere soil of a healthy tomato plant in Bulgaria

Yoana Kizheva^{a,*}, Melani Eftimova^a, Radoslav Rangelov^a, Neli Micheva^a, Zoltan Urshev^b, Iliyana Rasheva^a, Petya Hristova^a^a Sofia University St. "Kliment Ohridski", Faculty of Biology, Department of General and Industrial Microbiology, 8 Dragan Tsankov Blvd, Sofia 1000, Bulgaria^b LB Bulgaricum Plc., R&D Department, 14 Malashevskva Str., Sofia, Bulgaria

ARTICLE INFO

Keywords:

Broad host range bacteriophages
Bacterial spot disease
Xanthomonas vesicatoria
Phage biocontrol
Podoviridae phages

ABSTRACT

The urgent need of research of new approaches to control bacterial disease on economical important crops, focuses our attention on bacteriophages as alternative biocontrol agents. Thus, the purpose of this paper is to present the isolation and initial characterization of three bacteriophages (SfXv124t/1, 2 and 3) isolated from rhizosphere soil of a healthy tomato plant in Bulgaria that are capable to lyse three phytopathogenic bacteria. The initial characterization includes determination of: their host range, plaque morphology, optimal storage temperature of pure phage lysates, their sensitivity to UV light, thermal inactivation, optimal multiplicity of infection (MOI) and virion morphology. The obtained results showed that one of the phage isolates was capable to lyse wild strains from three phytopathogenic bacterial species: *Xanthomonas vesicatoria*, *Xanthomonas euvesicatoria* and *Xanthomonas gardneri*, and the two remaining phages were active against *X. vesicatoria* and *X. euvesicatoria*. On *X. vesicatoria* lawn, the phages produced the same plaque types that differed only in their size. Storage at 4 °C for 26 days did not lead to decrease in phage titer as opposed to storage at 28 °C followed by decrease to varying degree for all three phages. The results obtained after exposure of the phage lysates to sunlight (UVA + B) and UVC light in separate experiments showed that UVC had a potent phagocidal effect as after 50 min of exposure there were no viable phages in the samples. UVA an UVB had lethal effect for two of the phage isolates and absolutely no lethal effect for the third one as after 50 min of exposure to sunlight there was no decrease in the initial phage titer. Phage isolates were tested for their thermal inactivation after incubation of pure phage lysates at three different temperatures: 55 °C, 75 °C and 95 °C for a period of 10 and 30 min. The most lethal temperature turned out to be 95 °C as after 10 min there were no viable phages in the samples. Phage isolate SfXv124t/1 was the most susceptible as its titer decreased by 1 lg after 10 min of incubation at 55 °C and by another 1 lg after 30 min. The most thermally resistant isolate was SfXv124t/3 as its titer remained stable after 30 min of incubation at 55 °C and decreased only by 1 lg after incubation at 75 °C for 10 min. The optimal MOI for SfXv124t/3 was 0,01 (tested range 0,01–100) with maximal phage titer, reported at the 24th hour of incubation. TEM micrographs of the same isolates reveals that it belongs to family Podoviridae.

1. Introduction

Tomatoes (*Solanum lycopersicum* L.) and sweet pepper (*Capsicum annuum* L.) are among the most consumed vegetables all over the world. One of the main reasons for great losses of production in the agriculture especially in countries with worm and humid climate are bacterial diseases on crops. According to scientific opinion of EFSA Panel on Plant Health these losses can reach up to 30%. Bacterial spot disease is wide spread in Europe as among the most affected countries are Serbia (Obradovic et al., 2004), Turkey (Aysan and Sahin, 2003), Republic of

North Macedonia (Mitreva and Kovacevic, 2006), Italy (Buonaurio and Stravato, 1992) and Spain (Melgarejo et al., 2010). To date there are several bacterial species which are regulated harmful organisms in the EU (European Union), and thence in Bulgaria as a member of the union (EFSA, Scientific Opinion, 2014). Four phytopathogenic xanthomonad species (*X. vesicatoria*, *X. euvesicatoria*, *X. gardneri* and *Xanthomonas perforans*) are considered to cause the bacterial spot disease. They can infect either tomato or pepper, or both plants, simultaneously (Jones et al., 2004; Vauterin et al., 1995). These bacterial species are stated among the tenth most economically important bacterial species in agriculture in

* Corresponding author.

E-mail addresses: joana_k@biofac.uni-sofia.bg, yokizheva@gmail.com (Y. Kizheva).

countries with warm and humid climate (Mansfield et al., 2012). The rapid spread of the pathogens in the fields, greenhouses and across country borders is due to infected tomato and pepper seeds, wind, rains and cultivation practices.

For the last nearly 30 years prof. Bogatsevska and coworkers have had a great contribution to the study of bacterial-spot-causing xanthomonads in Bulgaria as they isolated and characterized a large number of strains from infected tomato and pepper plants (Bogatsevska and Sotirova, 1992; 2022). The major bacterial spot causative agents on tomato plants in Bulgaria are *X. vesicatoria* followed by *X. gardneri* and on pepper plants - *X. euvesicatoria* (Kizheva et al., 2011, 2013; Vancheva et al., 2018). The first report of *X. euvesicatoria* isolated from infected tomato plants in Bulgaria was made in 2020 (Kizheva et al., 2020).

Disease control is difficult due to the increasing resistance of the causative agents to copper pesticides and antibiotics (Jones et al., 2012; Marco and Stall, 1983; Minsavage et al., 1990; Pernezny et al., 2008; Thayer and Stall, 1961). Furthermore, the excessive use of chemical pesticides leads to accumulation of different pesticides residues in fruit, meat, animal feed and environment (Chourasiya et al., 2015; EFSA, 2020; Witzak and Abdel-Gawad, 2014), which can cause various harms in the human and animal bodies, such as dermatological, respiratory, neurological (Alewu and Nosiri, 2011; Nicolopoulou-Stamati et al., 2016; Sanborn et al., 2007; WHO, 1990) and even death (Gunnell et al., 2007). All that leads to one main conclusion: the urgent need of an alternative manner for fighting bacterial diseases in agriculture, such as phage therapy. One of the growing phenomena in phage therapy, not only in the agriculture but in the human and animal medicine too, is the use of phage cocktails. These are mixtures of several phage types, having different host ranges, in order to be effective against different strains of the target bacterium and moreover, to be effective against more than one bacterial species (Chan and Abedon, 2012; Gill and Hyman, 2010; Goodridge, 2010). According to Ross et al. (2016) phage preparations are more effective when a small number of broad host range phages are included in the mixtures, instead of great number of narrow host range phages.

As potential biocontrol agents, phages have to meet certain criteria, such as: 1/obligately lytic life cycle; 2/lack of genes for toxins; 3/broad host range; 4/to stay viable for long time at different storage temperatures (Ackermann et al., 2004; Hyman, 2019). There are some additional factors that could have negative effect on phages when applied in environmental conditions (field application for example) and which have to be screened for newly isolated biotherapeutic phages. Such environmental factors could be: sunlight irradiation, pH, temperature, humidity, ability to survive in phyllosphere and rhizosphere and sensitivity to chemical pesticides (Iriarte et al., 2007; Jones et al., 2007; Suttle and Chen, 1992; Svircev et al., 2010). Full characterization of a newly isolated bacteriophage intended for use as a biocontrol agent include some additional characteristics such as optimal MOI, determination of plaque and virion morphology, whole genome sequencing etc., which are not directly related to phage characteristics in phage therapy application (Hyman, 2019).

The original idea of this study, was the isolation of *X. vesicatoria* effective lytic bacteriophages as it is one of the most common phytopathogenic species on tomato plants in Bulgaria. However, in this paper we present for the first time, the isolation and initial characterization of three broad host range bacteriophages isolated from rhizosphere soil of a healthy tomato plant capable of infecting the three wild type bacterial-spot-causing xanthomonad species, widespread in Bulgaria. These phage isolates could have a potential as biocontrol agents against bacterial spot disease on tomato and pepper plants.

2. Materials and methods

2.1. Bacterial cultures and cultivation conditions

The bacterial species and strains used in this study are selected from a large collection of xanthomonad phytopathogenic bacteria, collected for a period of 22 years from prof. Nevena Bogatsevska and coworkers and

kindly donated to the Department of General and Industrial Microbiology, Faculty of Biology, Sofia University "St. Kliment Ohridski". They differ in their year and location of isolation, pathotype and races (Table 1). All strains were previously identified and characterized (Kizheva et al. 2013, 2020; Bogatzevska and Sotirova, 2002). The following type cultures were used as controls in the host range determination: *Xanthomonas euvesicatoria* NBIMCC 8731, *Xanthomonas perforans* NBIMCC 8729, *Xanthomonas vesicatoria* NBIMCC 2427, *Xanthomonas gardneri* NBIMCC 8730 and *Pseudomonas syringae* pv. *tomato* NBIMCC 3374. LB broth (Luria Bertani) was used for phage lysate preparation, Nutrient agar (NA) containing beef extract 3 g/L, peptone 5 g/L and agar 15 g/L was used for plaque assays and PSA (potato sucrose agar) was used for obtaining of log-phase bacterial cultures.

2.2. Sample collection and isolation of bacteriophages

For bacteriophages isolation a tomato rhizosphere soil sample was used which was collected during late summer from a self-grown healthy tomato plant in the city of Sofia, Bulgaria. The methodology of isolation was as previously described by Ahmad et al. (2014) and Yamada et al. (2007). The presence of lytic phages in soil sample was detected via double agar overlay plaque assay (DAOPA) and as host bacteria 11 *X. vesicatoria* strains (29t, 1b, 60t, 68t, 73t, 97t, 131t, 132t, 124t, 130t and 41f) were used (Table 1.). The soil sample (10g) was placed in sterile centrifuge tube and filled with sterile distilled water to the cap (approximately 30 mL). The sample was stored at room temperature for 30 min with gentle inversions every 5 min. Next, the sample was centrifuged at 8000 g for 20 min and the supernatant was filtered through a 25 mm sterile membrane filter (0.2 µm pore size, Corning syringe filters, USA) for removing any residues of bacterial cells. Aliquot of 100 µL of the filtered supernatant was mixed with: 1) 100 µL host bacterial suspension (10⁸ cfu/ml) obtained from overnight bacterial culture, suspended in saline; 2) 30 µL 1M CaCl₂ and 3) 3 mL soft NA containing 0.45% agar. All ingredients were mixed well by vortexing for 10 s and quickly poured onto the surface of previously prepared 1.5% agar plate. The double agar plates were then incubated at 28 °C for 24 h and positive outcomes were scored after establishment of clear plaques from bacteriophages as a result of bacterial lysis.

2.3. Phage purification and characterization

Pure bacteriophage cultures were obtained after propagation and purification of phages. The bacterial culture used as specific host for phage propagation was *X. vesicatoria* strain 124t (tomato isolate) grown overnight (log phase). Aliquot of 100 µL of the bacterial suspension (0.5 McFarland scale – about 10⁸ CFU/mL) was inoculated in 50 mL LB broth in 500 mL flasks. A single phage plaque was isolated and added by dint of microbiological needle and 0.5 mL 1M CaCl₂ was added. The flasks were cultivated on rotary shaker overnight. The media broth was then centrifuged at 8000g for 20 min and the supernatant containing viral particles was filtered through a sterile membrane filter to remove any residues of bacterial cells. The cell-free supernatant was 10-fold diluted in sterile saline and sub cultured with the target bacterium (as described above) three consecutive times for phage purification. Pure phage cultures were stored for further studies at 4 °C as pure phage lysates.

2.3.1. Host range determination

The host range of the three newly isolated bacteriophages was determined by testing the sensitivity of 23 phytopathogenic bacterial strains to the phages in three independent trials: *X. vesicatoria* (14 strains), *X. euvesicatoria* (5 strains), *X. gardneri* (3 strains) and one *P. syringae* pv. *tomato* strain (Table 1). Five type cultures were also included in the assay (p. 2.1.). Spot testing assay was applied (Kutter, 2009). All bacterial cultures were grown on PSA overnight (16–20 h) and subsequently suspended in sterile saline. The bacteriophage lysates were obtained after cultivation of each phage separately, with *X. vesicatoria* strain 124t as host in 50 mL LB broth for one night. One hundred

Table 1. Phytopathogenic bacteria used as specific hosts for phages isolation and host range determination.

№	Bacterial hosts					Phage isolates		
	Strain	Pathotype	Race	Year of isolation	Location	SfXv124t/1	SfXv124t/2	SfXv124t/3
<i>X. vesicatoria</i>								
1.	29t	PT	T3	1995	Petrich	+	+	+
2.	31t	T	T1	1997	Sofia	–	–	–
3.	1b	PT	T1	1999	Lovech	–	+	–
4.	32t	PT	T3	1999	Sofia	–	–	–
5.	43t	PT	T2	2006	Kostinbrod	–	–	–
6.	60t	T	T2	2007	Sofia	–	–	–
7.	68t	T	T3	2010	Plovdiv	–	+	–
8.	73t	T	T1	2012	Topolovgrad	–	+	+
9.	97t	PT	T2	2015	Plovdiv	–	–	–
10.	131t	T	T2	2016	Blagoevgrad	+	+	+
11.	132t	PT	T1	2016	Tulenovo	+	+	+
12.	124t	T	T3	2016	MVCRI, Plovdiv*	+	+	+
13.	130t	T	T2	2016	Blagoevgrad	–	+	+
14.	41f	NS	NS	2017	Plovdiv	+	+	+
15.	**NBIMCC 2427	NA	NA	NA	NA	–	–	–
<i>X. euvesicatoria</i>								
16.	99b	PT	T3	2013	Byala Cherkva	+	+	+
17.	108t	PT	T2	2015	Svilengrad	+	+	+
18.	269p	P	P4	2015	Durankulak	+	–	+
19.	274p	P	P0	2015	Shabla	+	–	+
20.	105t	PT	T1	2015	Haskovo	+	–	+
21.	NBIMCC 8731	NA	NA	NA	NA	–	–	–
<i>X. gardneri</i>								
22.	62t	PT	T1	2009	Sofia	+	–	–
23.	64t	PT	T3	2009	Sofia	–	–	–
24.	77t	T	T3	2012	Topolovgrad	+	–	+
25.	NBIMCC 8730	NA	NA	NA	NA	–	–	–
<i>X. perforans</i>								
26.	NBIMCC 8729	NA	NA	NA	NA	+	–	+
<i>Pseudomonas syringae</i> pv. <i>tomato</i>								
27.	32f	NS	NA	2017	NS	–	–	–
28.	NBIMCC 3374	NA	NA	NA	NA	–	–	–

“+” – sensitive strain, plaques formation; “–” – resistant strain, no plaques formation; NA – not applicable; NS – not screened.

*MVCRI – Maritsa Vegetable Crops Research Institute, Plovdiv, Bulgaria; **NBIMCC – National Bank for Industrial Microorganisms and Cell Cultures.

Bold font – *Xanthomonas vesicatoria* strains used as host bacteria during bacteriophage initial isolation from the soil sample.

microliters from each bacterial suspension (10^8 CFU/ml) were transferred in sterile tubes and mixed with 30 μ L 1M CaCl₂ and 3 mL soft NA with agar concentration of 0.45%. The mixture was then added onto the main agar layer in petri dishes prepared the day before and left at room temperature for complete solidification. The phage lysates were centrifuged at 8000 rpm/min for 20 min and filtered through membrane filter (0.22 μ m pore size) to completely remove the residual bacterial cells. The pure phage lysates were 10-fold diluted in sterile saline and each dilution was applied onto double agar lawn of every bacterial strain via spots (about 10 μ L). The petri dishes were cultivated at 28 °C for 20 h. The positive outcomes were scored after the establishment of clear plaques from bacteriophages as a result of lysis of sensitive bacteria (Msimbira et al., 2016; Pringsulaka et al., 2011).

2.3.2. Characterization of phage plaques

The type of the plaques which occurred on the agar surface after bacterial lysis from the three phages was determined by measuring the plaque diameter and establishing the presence/absence of halo. This characterization was done by cultivating the phages along with their specific host (*X. vesicatoria* strain 124t) on solid agar media via DAOPA. The morphology of the plaques was determined in accordance with Jurczak-Kurek et al. (2016).

2.3.3. Determination of phages survival at two storage temperatures

Pure phage lysates were stored at 4 °C and 28 °C for a period of 26 days. The choice of these two storage temperatures was based on the ability to store a phage biopesticides under non-specific conditions. Phage survival at these storage conditions was determined by tracking the change in phage titer after 18, 22 and 26 days of storage. Spot testing assay was applied for determination of phage titers.

2.3.4. Thermal inactivation of phage isolates

Phages thermal inactivation after incubation at three different temperatures for a period of 10 and 30 min was determined according to Gasic et al. (2011) and Jurczak-Kurek et al. (2016), with minor modifications. Pure phage lysates (2 mL) were incubated at three temperatures: 55 °C, 75 °C and 95 °C in Dry Block Thermostat TDB-100 (BOECO Germany). After every time point, 1mL from each phage lysate was 10-fold diluted in sterile saline and 10 μ L from every dilution were spotted on *X. vesicatoria* strain 124t lawn in petri dishes. The number of viable phages after thermal inactivation was calculated by counting the plaques formed on bacterial lawn following incubation at 28 °C for 24h. and subsequent comparison with the initial titer of the phages. The experiment was done three times and the average values were calculated.

2.3.5. Sensitivity of the newly isolated phages to sunlight (UVA + B) and UVC light

The effect of different types of UV light on phages viability was established. The effect of UVA + B ($\lambda = 280\text{--}400\text{ nm}$) was screened by direct sunlight irradiation (petri dishes were opened) during summer season (year 2019) in bright and sunny days without any clouds in the sky and the effect of UVC light ($\lambda = 253,7\text{ nm}$) - by constant UV output irradiation in closed box in the laboratory. The results were interpreted by counting the numbers of viable phages capable to form plaques on host lawn before and after irradiations. The assay was done in accordance with Tomlinson (2010), with minor modifications. The initial phage titers were determined after 10-fold dilution via Spot testing assay. Five milliliters of each phage suspension/lysate were placed in sterile opened petri dishes and directly irradiated for a period of 10, 30 and 50 min. When irradiated by UVC light, the samples were placed in a closed box (L88 cm/W60 cm/D63 cm) equipped with UVC lamp (PHILIPS UVC, TUV 15W/G45T8). The lethal effect of UV light on phages was established by measuring the difference between the initial number of the viable phages in the suspensions and their number following irradiation. The final results were obtained after three separate trials were taken into account.

2.3.6. Phage SfXv124t/3 – determination of virion morphology and optimal MOI

2.3.6.1. Transmission electron microscopy. Phage isolate SfXv124t/3 was selected for performance of TEM in order to obtain the virion morphology. Phage was propagated 24h via DAOPA using *X. vesicatoria* strain 124t as specific host for obtaining clear plaques. A high phage titer solution was made using 5 mL freshly prepared phage buffer (10 mM Tris – HCl; 10mM MgSO₄; 68 mM NaCl; 1 mM CaCl₂) flooded onto the agar surface (Msimbira et al., 2016). The petri dish was stored at room temperature for at least 2 h with gentle rotations of the dish every 10–15 min. Phage buffer, containing viral phages extracted from the plaques by rotations, was carefully removed from the petri dish using sterile syringe and filtered through sterile membrane filter. Fifty μL from the resulted phage solution was dripped on formvar-coated electron microscopy grids and then negatively stained with 1% uranyl acetate in 70% methanol (Borisova et al., 2018). The observation was examined with JEOL JEM 2100 apparatus operating at 200 kV (JOEL, Japan). The size of phage capsid was calculated as the average value from three independent images.

2.3.6.2. Determination of optimal MOI of phage isolate SfXv124t/3. In order to establish the most appropriate MOI for phage propagation five different ratios were tested (0.01, 0.1, 1, 10 and 100), according to Gasic et al. (2011) and Pringsulaka et al. (2011), with minor modifications. The assay was done by mixing: 1/1mL bacterial suspension containing different number of cells, according to different MOI (MOI 0,01–10⁸ cfu/mL, MOI 0,1–10⁷ cfu/mL, MOI 1–10⁶ cfu/mL, MOI 10–10⁵ cfu/mL and MOI 100–10⁴ cfu/mL); 2/1mL phage lysate (10⁶ PFU/mL); 3/0.5 mL 1M CaCl₂; and 4/50 mL LB broth in 100 mL flask. The flasks were incubated on a rotary shaker at 28 °C for 3h, 6h and 24h. Phage titers were measured at 3h, 6h and 24h for every MOI. The number of viable phages were measured after tenfold dilutions via Spot testing assay. The

optimal MOI was considered as highest phage titer obtained after three separate trials.

3. Results

3.1. Isolation of bacteriophages

The phages were isolated from rhizosphere soil sample, collected from symptomless self-grown tomato plant in Sofia, Bulgaria. The plant had not been treated with any pesticides, whether copper chemicals or other. Three bacteriophages were collected from three randomly selected clear plaques formed after 24h cultivation of the soil sample with the target bacterium - *X. vesicatoria* strain 124t (tomato isolate). The phages were isolated as described above and named as follows: SfXv124t/1, SfXv124t/2 and SfXv124t/3. For obtaining pure phage cultures, the new isolates were purified as described above (p. 2.3.).

3.2. Host range determination

Wild type phytopathogenic bacteria (23 strains) and 5 reference cultures were used for phages host range determination. Phage isolates SfXv124t/1 and SfXv124t/3 had lytic activity against wild host strains belonging to the bacterial species *X. vesicatoria*, *X. euvesicatoria* and *X. gardneri* to varying degree. These phage isolates were active also against *X. perforans* NBIMCC 8729. Phage isolate SfXv124t/2 had lytic activity against wild host strains belonging to species *X. vesicatoria* and *X. euvesicatoria* and none against the type cultures tested. Out of 23 tested wild type phytopathogenic isolates 52% were sensitive to the phage SfXv124t/1, including 5 *X. vesicatoria* strains, 5 *X. euvesicatoria* strains and two *X. gardneri* strains (Table 1). The sensitive strains to phage isolate SfXv125t/2 were 48%, which means it has lower activity than SfXv124t/1, but it differed in host range at strain level form SfXv124/1. SfXv124t/2 was active against 9 *X. vesicatoria* strains and only 2 *X. euvesicatoria* strains. The host range of the third phage SfXv124/3 had lytic activity against 57% of tested bacterial strains, which makes it the most effective isolate. Among the sensitive strains were 7 *X. vesicatoria* strains, 5 *X. euvesicatoria* strains and one *X. gardneri* strain. Interestingly, all type cultures tested, including *P. syringae* pv. *tomato*, were not sensitive to the phage activity, with one exception (*X. perforans* NBIMCC 8729) mentioned above.

3.3. Characterization of phage plaque morphology on agar media

The three phage isolates formed relatively small plaques on soft NA after cultivation with *X. vesicatoria* strain 124t as host bacterium (Figure 1). The greatest plaque diameter was observed for phage isolate SfXv124t/1 (about 2 mm). The other two isolates (SfXv124t/2 and SfXv124t/3) formed plaques with diameters of about 1 mm. All plaques were clear, without halo.

3.4. Determination of phages survival at two storage temperatures

The effect of two storage temperatures (4 °C and 28 °C) of pure phage lysates was tracked for 26 days. The results showed that there was no

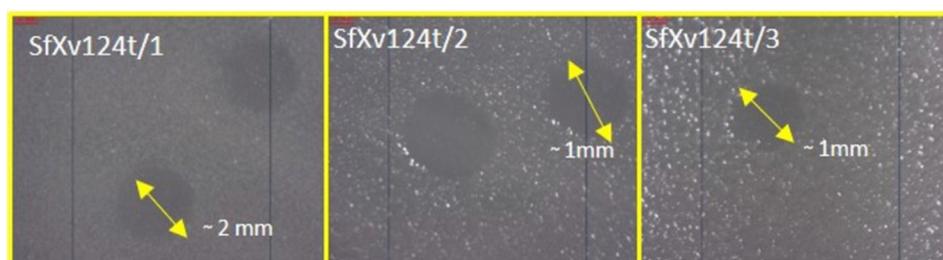


Figure 1. Clear plaques formed by the three newly isolated bacteriophages on NA plates; lawn: *X. vesicatoria* strain 124t used as specific bacterial host.

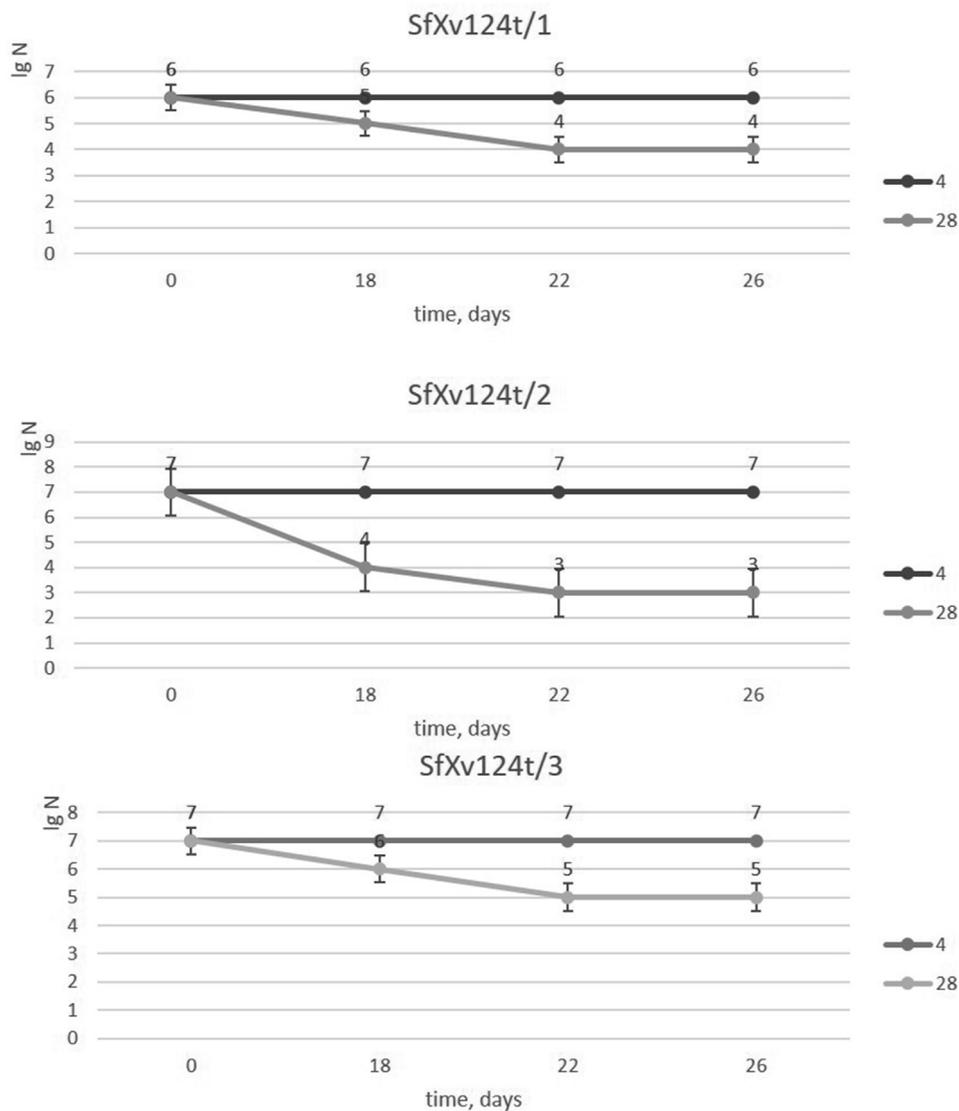


Figure 2. Phage viability after storage at 4 °C and 28 °C as pure lysate for a period of 26 days. The presented results show the averages of three independent studies.

decrease in the titers of all three phage isolates after storage at 4 °C. The storage of the lysates at 28 °C led to a decrease in phage titers to varying degree. The most unstable phage isolate turned out to be SfXv124t/2 as its titer decreased by 4 lg after 26 days as opposed to the other two, SfXv124t/1 and SfXv124t/3, each with only 2 lg decrease in titers after 26 days of storage at 28 °C (Figure 2).

3.5. Thermal inactivation of phage isolates

The complete thermal inactivation of the three tested phages occurred after 10 min of incubation at 95 °C as under these conditions there were no viable phages in the samples. The viability of the phages, however, stayed relatively stable after incubation at 55 °C and 75 °C for 10 and 30 min (Figure 3). SfXv124t/3 proved to be the most heat-resistant isolate as its titer did not decrease after 30 min at 55 °C and only 1 lg and 3 lg drops in titer were observed after 10 and 30 min at 75 °C, respectively. Phage isolate SfXv124t/2 also showed good heat resistance but to a lesser degree than SfXv124t/3, since its titer dropped by 5 lg after 30 min at 75 °C. Phage isolate SfXv124t/1 was the most sensitive to high temperature as its titer started to decrease in only 10 min at 55 °C and there were almost no viable phages in the sample following 10-min incubation at 75 °C.

3.6. Sensitivity of the newly isolated phages to sunlight (UVA + B) and UVC light

The exposition of pure phage lysates from the three phages to direct sunlight (UVA + B) for 50 min showed that phage isolate SfXv124t/3 was the most stable as its titer did not decrease after the irradiation period. Phage isolate SfXv124t/1 also showed good stability as its titer stayed stable to the 30th minute of the experiment and decreased only by 1 lg after 50 min. The most sensitive isolate proved to be SfXv124t/2. Phage titer stayed stable to the 10th min of exposure to sunlight. The number of viable phages in the sample decreased by 2 lg after 30 min and another 2 lg at 50th min, respectively. Lethal effect was observed after irradiation with UVC light. The concentration of viable phages in the samples decreased at varying degree for each isolate. After 30 min of UVC irradiation there were still viable phages able to lyse their host bacteria and to form plaques, but after 50 min no viable phages were detected in the samples (Figure 4).

3.7. Determination of virion morphology and optimal MOI of phage SfXv124t/3

Phage isolate SfXv124t/3 was chosen for additional analysis (TEM and determination of optimal MOI), since it was the most appropriate

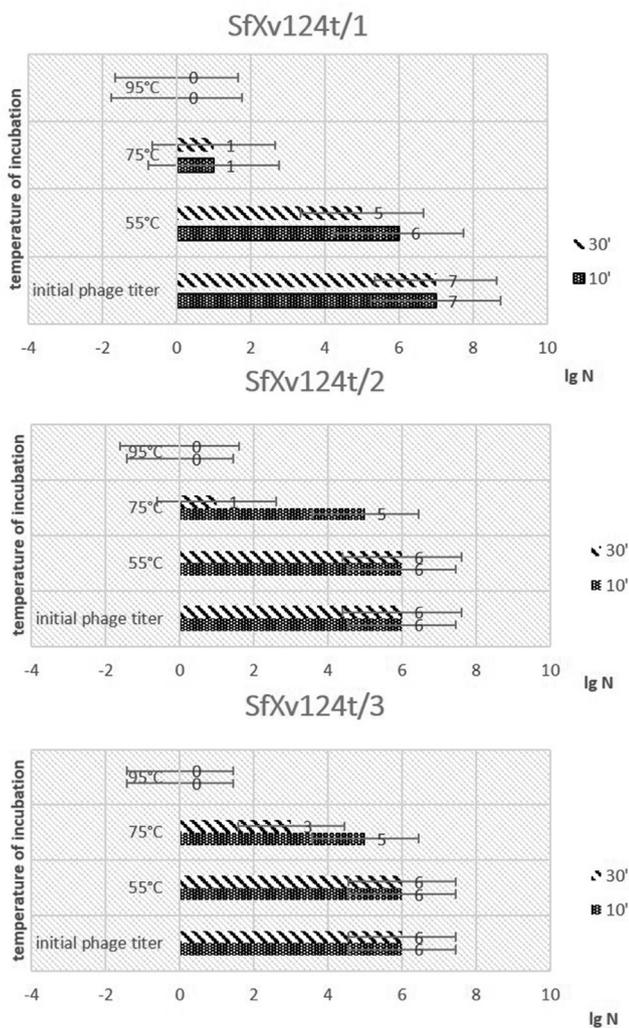


Figure 3. Thermal inactivation and survival of the phage isolates at 55 °C, 75 °C and 95 °C.

isolate as biocontrol agent against bacterial spot disease. According to TEM micrographs, phage isolate SfXv124t/3 had icosahedral capsid and short tail (Figure 5). The head diameter was below 50 nm. These results clearly placed this phage isolate within order *Caudovirales* (tailed phages), family *Podoviridae* (Ackerman, 1999) or Group C according to Bradley classification (Bradley, 1967). The isometric shape of the head placed the phage in group C1 (Ackermann and DuBow, 1987).

The optimal MOI for further phage propagation was established after cultivation of phage SfXv124t/3 (10^6 PFU/mL) with the target bacterium *X. vesicatoria* strain 124t for a period of 24h. In this experiment we have found out that, following 3h and 6h of incubation, there were drops in phage titers at every MOI tested, in different ratios. The greatest number of viable viral particles (i.e. the highest phage titer) in the samples was detected after cultivation at MOI 0.01 (10^8 PFU/ml) for 24h, hence we accepted these conditions of cultivation as optimal. The assay was performed in three separate trials and the average values of phage titers, obtained at every stage of the experiment, were calculated.

4. Discussion

Bacterial spot on tomato and pepper is one of the most devastating diseases in Bulgaria. The causative agents which have been isolated in the country are *X. vesicatoria*, *X. euvesicatoria* and *X. gardneri*. Up to now there is no data for *X. perforans* being isolated from infected tomato and pepper plants in Bulgaria. There are many papers that are focused on the

promising potential of phage therapy application for fighting different bacterial diseases in crop production (Ahmad et al., 2014; Chae et al., 2014; Civerolo and Keil, 1969; Gasic et al., 2018; Schwarczinger et al., 2017; Yamada et al., 2007).

In nature each pest has its antagonist as is the case of phytopathogenic bacteria and their bacteriophages. Studies focused on isolation and characterization of such bacteriophages aim to mobilize their potential as means of pest control. In the current study phytopathogenic bacteria causing bacterial spot disease on tomato and pepper were challenged by newly isolated bacteriophages that can infect them. For developing an effective mixture of lytic phages for disease management it is necessary that phages infecting all the three bacterial spot disease causative agents to be present in the preparation. Phages effective against different *Xanthomonas* sp. species were previously reported, since in most cases they were narrow host range phages i.e. able to lyse strains of one bacterial species (Ahmad et al., 2014; Gasic et al., 2018) and in one case – a broad host range *X. perforans* phage capable of lysing strains of two bacterial species: *X. perforans* and *Xanthomonas citri* subsp. *citri* (Balogh et al., 2018). The reason for choosing *X. vesicatoria* as specific host for phage isolation is that it is the main bacterial spot causative agent in tomatoes and we couldn't find any reports for isolation and characterization of *X. vesicatoria* effective phages. There is only one study that reports a novel *X. vesicatoria* filamentous phage isolated from pepper field in Mexico but it is a short announcement and is focused mainly on the genome organization of the phage without presenting details concerning phage characterization (Solis – Sanchez et al., 2020).

In the majority of cases lytic phages have been isolated from the soil samples around roots of infected plants (Gasic et al., 2018; Nagy et al., 2011). In our case lytic phages were isolated from rhizosphere of a healthy plant that had not been treated with any chemical pesticides. We randomly selected three phage isolates from clear plaques formed in the petri dish with *X. vesicatoria* strain 124t (tomato isolate) as host bacterium. Plaques indicating bacterial lysis were also observed on petri dishes with the rest of the ten *X. vesicatoria* host strains used in the initial isolation but they were not as clear enough as those, obtained with strain 124t.

The highest number of potential host bacterial strains for phage host range analysis were selected among the *X. vesicatoria* strains in our collection as in the beginning of the experiments we expected that the phages would be effective primarily against strains from the species used for their initial isolation (*X. vesicatoria*). All tested strains (23) however were previously identified (Bogatsevskaja and Sotirova, 1992; Kizheva et al., 2011, 2013, 2016; Vancheva et al., 2018) and their pathogenicity and race structure were established. Surprisingly, we found out that the newly isolated phages had broader host range (p. 3.2.). Based on these results, we could classify them as broad host range bacteriophages due to their ability to lyse three closely related but different bacterial species (Greene and Goldberg, 1985; Khan and Nilsson, 2015; Uchiyama et al., 2008; Yu et al., 2016).

Among the xanthomonad bacteria tested in this study, there were several strains susceptible to the three phages simultaneously (7 strains; Table 1) and several strains that were resistant to all of the three phages (6 strains; Table 1). For rest of the tested bacterial strains the sensitivity to the three bacteriophages varies at strain level. There are several possible explanations of such strain-specific interaction at species level – differences in the cell wall structure of these bacteria and particularly, phage receptors localized in the outer membrane (proteins and LPS structure), production of exopolysaccharides, differences in bacterial defense systems (CRISPR-Cas, RM systems, Abi) or action of Sie (superinfection exclusion) systems. At this stage of the research we could not exactly clarify the reason for this selective interaction between phages and bacteria. No correlation between phage sensitivity and the pathotype or the race of the phytopathogenic bacteria could be found, because there were representatives of all pathotypes (Tomato (T), Pepper-tomato (PT) and Pepper (P)) and tomato races (T1, T2 and T3) among resistant and sensitive bacterial strains, respectively. Similar to our results showing

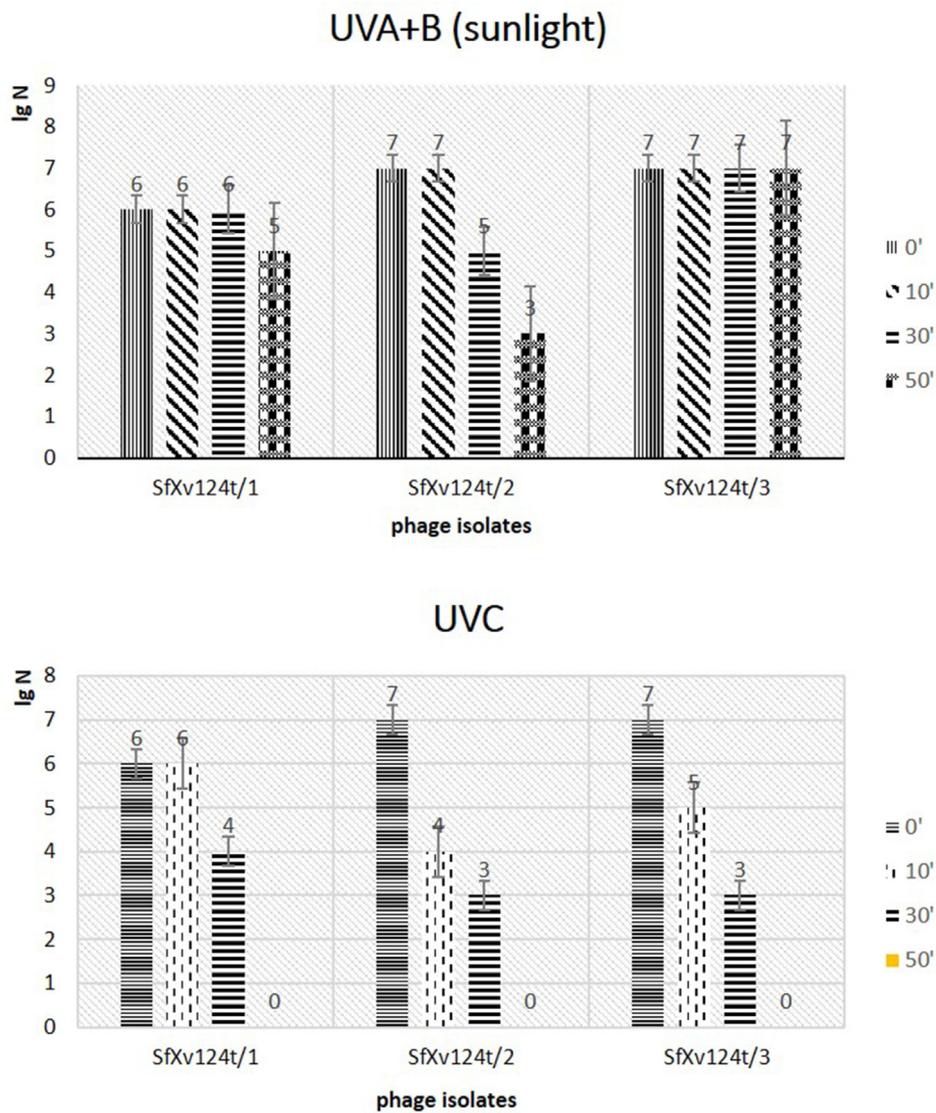


Figure 4. Effect of sunlight (UVA + B) and UVC irradiation on the newly isolated phages.

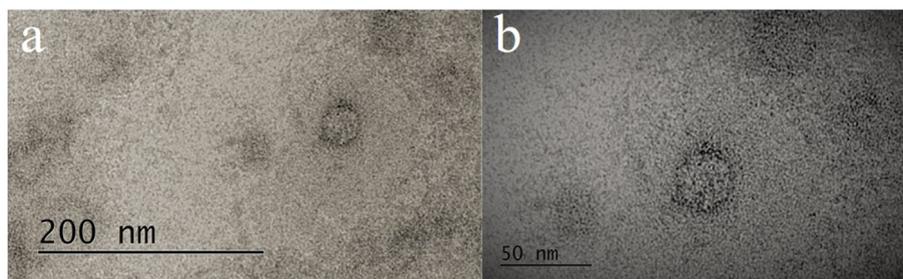


Figure 5. TEM micrograph of the phage isolate SfXv124t/3. The image shows the shape of the capsid and a short tail. Bars, 200 nm (a) and 50 nm (b).

differences in phage activity in strain level had been reported before for phages effective against phytopathogenic bacteria *R. solanacearum*, *Erwinia amylovora* and *X. euvesicatoria* (Bouzar et al., 1999; Kalpage and De Costa, 2014; Schwarczinger et al., 2017).

The long storage stability of phage preparations (biopesticides for example) intended for personal use under standard conditions (fridge or room temperature) is essential to be tested due to impossibility to be stored under special conditions such as $-20\text{ }^{\circ}\text{C}$, $-70\text{ }^{\circ}\text{C}$ etc. In our study we found that phage isolates did not lose viability when stored at $4\text{ }^{\circ}\text{C}$ for

almost a month. These results correlate with those reported by Gasic et al. (2018) for *X. euvesicatoria* specific phages and those reported by Clark (1962) who concluded that $4\text{ }^{\circ}\text{C}$ was the most suitable temperature for pure phage lysate storage. Therefore, a potential phage preparation will be easily stored. The results obtained after storage at $28\text{ }^{\circ}\text{C}$ showed that this temperature was not appropriate due to decrease in efficiency of plating of the isolates at varying degree for each of them. These findings are also in compliance with the results reported in the literature (Clark, 1962; Gasic et al., 2018).

The thermal inactivation of a phage could be discussed in two directions. In dairy industry for example, it is essential for phages to be thermally unstable (Pringsulaka et al., 2011). However, in other biotechnological industries, such as phage biopesticide production process for example it could be good to know phage thermal inactivation temperature in order to avoid it. The three phages reported show a good potential to stay viable and to be capable to lyse the target bacterium cells to varying degree after incubation at high temperatures. One of the phage isolates (SfXv124t/3) showed high temperature resistance (30 min at 75 °C) compared to other phages effective against *X. euvesicatoria* which were completely inactivated after 10 min at 70 °C–71 °C (Gasic et al., 2011).

Sensitivity to different environmental factors should also be considered, since phage preparations intended for use in pest control are applied directly in the field or other open spaces. One of the environmental factors that might have lethal effect on bacteriophages is sunlight (UVA + B light) and hence it might reduce their effectiveness. Our results (p.3.6.) were in good compliance with the observations of Iriarte et al. (2007), although in their experiments phage formulations were sprayed onto the tomato leaf surface where additional factors, such as wind, humidity, stability at the leaf surface, have essential role in phage survival and effectiveness. However, our findings for stability after direct UVA + B irradiation may serve as a good basis for further analysis. The UVC ($\lambda = 253.7$ nm) irradiation had expected greater lethal effect on the three bacteriophages. It is well known that UVC irradiation have great bactericidal, virocidal and phagocidal effect. It is important that newly isolated biological agents (such as bacteriophages) to be screened for resistance to one of the most widely applied sterilization methods, such as UVC irradiation. Nevertheless, all the three phages showed some resistance to UVC light as after 30 min of irradiation viable phages were still detected in the samples. Similar stability to UV light irradiation is reported for phages effective against *X. euvesicatoria* (Gasic et al., 2018) and *Xanthomonas campestris* pv. *vesicatoria* (Iriarte et al., 2007).

According to the phage plaque morphology and the classification of phage plaque types given from Jurczak-Kurek et al. (2016), we could conclude that the isolates are obligate lytic phages due to clear plaques (without halo) they formed onto solid agar plate. Plaques formed by the new isolates were relatively small. According to the same report, this kind of plaques are formed by members of family *Myoviridae* which have larger capsids. As opposed to them, *Siphoviride* and *Podoviride* phages have smaller capsids and formed larger plaques (Jurczak-Kurek et al., 2016). Our results did not completely correspond to aforementioned characteristics as the newly isolated phage formed small plaques but according to TEM micrographs they belong to family *Podoviridae*.

Phage effective against various members of the genus *Xanthomonas* have been previously reported. Among them are: 1/*Xanthomonas axonopodis* pv. *citri* - a filamentous *Inoviridae* phage (XacF1) (Ahmad et al., 2014), 2/*X. euvesicatoria* - a long-tailed *Myoviridae* phage (Gasic et al., 2011), 3/*Xanthomonas campestris* - a *Myoviridae* phage (Liew and Alvarez, 1981), 4/*Xanthomonas pruni* - a *Myoviridae* phage (Civerolo, 1970) and 5/*X. vesicatoria* - a filamentous phage (Solís-Sánchez et al., 2020). The majority of them belongs to the group of tailed phages, which represent approximately 96% of all known phages. According to Ackermann this is the most common group of phages on Earth (Ackermann, 1999). Among them, the *Podoviridae* phages are the rarest (13.9%). Apart from that, there are several reports for such phages effective against different phytopathogenic bacteria as *Ralstonia solanacearum* phages (Bae et al., 2012), *Erwinia amylovora* phages (Schwarczinger et al., 2017) and *Pantoea agglomerans* phages (Adriaenssens et al., 2011). To our knowledge, our study presents for the first time a short-tailed phage isolated from tomato rhizosphere soil and effective against three wild type phytopathogenic bacterial species (*X. vesicatoria*, *X. euvesicatoria* and *X. gardneri*).

The ratio between the initial numbers of phage particles and bacterial cells during cultivation is defined as multiplicity of infection (Birge, 2000). The meaning and the concept of MOI could be discussed at

different perspectives: semantically, historically, mathematically, pharmacologically, practically and so on, depending on the objectives of the study (Abeldon, 2016). In the current study we discuss the practical concept of MOI in order to establish the best phage – bacteria ratio for further experimentations, production of high titer phage lysates and application. In our study no correlation was found between high MOI and higher phage yields, similar to other results reported by Hall et al. (2012). The highest phage titer (i.e. the optimal MOI) was observed at MOI 0.01 after 24 h of cultivation which corresponded to the results reported by Gasic et al. (2011) which established the same MOI rates as optimal.

5. Conclusion

Rhizosphere soil of a healthy tomato plant proved to be a suitable source for isolation of broadspectrum bacteriophages capable of infecting three closely related phytopathogenic bacteria causing serious disease on tomato and pepper plants - *X. vesicatoria*, *X. euvesicatoria*, and *X. gardneri*. In the current study we present that one of these phages belongs to family *Podoviridae*, which to our knowledge was not previously reported for phages effective against aforementioned phytopathogenic bacteria. This phage isolate (SfXv124t/3) also showed the highest effectiveness and robustness and therefore, it may be considered as potential candidate for a pest biocontrol agent, since out of the three characterized phage isolates it showed lytic activity against the largest number of wild strains (13 out of 23), the best tolerance to high temperatures and to UV light. The results described in this study could serve to enrich the knowledge of the natural diversity of the lytic phages effective against bacterial spot causing agents. The selected phage is a good candidate for further analyses concerning phage application as biocontrol agent.

Declarations

Author contribution statement

Yoana Kizheva: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Melani Eftimova, Radoslav Rangelov, Neli Micheva: Performed the experiments.

Zoltan Urshev, Petya Hristova: Analyzed and interpreted the data; Wrote the paper.

Iliyana Rasheva: Contributed reagents, materials, analysis tools or data.

Funding statement

This work was supported by the Bulgarian Ministry of Education and Science under the National Research Program "Healthy Foods for a Strong Bio-Economy and Quality of Life" approved by DCM # 577/17.08.2018".

Data availability statement

Data included in article/supp. material/referenced in article.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

- Abeldon, S.T., 2016. Phage therapy dosing: the problem(s) with multiplicity of infection (MOI). *Bacteriophage* 6 (3), 6 e1220348.

- Ackermann, H.-W., 1999. Tailed bacteriophages: the order *Caudovirales*. *Adv. Virus Res.* 51, 135–201.
- Ackermann, H.-W., DuBow, M.S., 1987. Viruses of Prokaryotes, 2. CRC Press, Boca Raton, FL, pp. 1–54, 55–161, 171–218.
- Ackermann, H.-W., Tremblay, D., Moineau, S., 2004. Long-term bacteriophage preservation. *World Fed. Cult. Coll. Newsl.* 38, 35–40.
- Adriaenssens, E.M., Ceysens, P.-J., Dunon, V., Ackermann, H.-W., Vaerenbergh, J.V., Maes, M., De Proft, M., Lavigne, R., 2011. Bacteriophages LIMelight and LIMEzero of *Pantoea agglomerans*, belonging to the “phiKMV-like viruses”. *Appl. Environ. Microbiol.* 77 (10), 3443–3450.
- Ahmad, A.A., Ogawa, M., Kawasaki, T., Fujie, M., Yamada, T., 2014. Characterization of bacteriophages Cp1 and Cp2, the strain typing agents for *Xanthomonas axonopodis* pv. *citri*. *Appl. Environ. Microbiol.* 80, 77–85.
- Alewu, B., Nosiri, C., 2011. Pesticides and human health. In: Stoytcheva, M. (Ed.), *Pesticides in the Modern World – Effects of Pesticides Exposure*. InTech, pp. 231–250. Available from: <http://www.intechopen.com/books/pesticides-in-the-modern-world-effects-of-pesticides-exposure/pesticide-and-human-health>.
- Aysan, Y., Sahin, F., 2003. Occurrence of bacterial spot disease, caused by *Xanthomonas axonopodis* pv. *vesicatoria*, on pepper in the Eastern Mediterranean region of Turkey. *Plant Pathol.* 52, 781.
- Bae, J.Y., Wu, J., Lee, H.J., Jo, E.J., Murugaiyan, S., Chung, E., Lee, S.-W., 2012. Biocidal potential of a lytic bacteriophage PE204 against bacterial wilt of tomato. *J. Microbiol. Biotechnol.* 22 (12), 1613–1620.
- Balogh, B., Thu, Nga N., Jones, J.B., 2018. Relative level of bacteriophage multiplication in vitro or in phyllosphere may not predict in planta efficacy for controlling bacterial leaf spot on tomato caused by *Xanthomonas perforans*. *Front. Microbiol.*
- Birge, E.A., 2000. *Bacterial and Bacteriophage Genetics*, fourth ed. Springer, New York, NY, USA.
- Bogatzevska, N., Sotirova, V., 1992. Occurrence of two pathotypes of *Xanthomonas campestris* pv. *vesicatoria* in Bulgaria. *TGC Report* 42, 11–12.
- Bogatzevska, N., Sotirova, V., 2002. Bacterial spot of tomato in Bulgaria: pathotypes and races. *Gene. Breed.* 31, 59–66.
- Borisova, D., Haladjova, E., Kyulavska, M., Petrov, P., Pispas, S., Stoitsova, S., Paunova-Krasteva, T., 2018. Application of cationic polymer micelles for the dispersal of bacterial biofilms. *Eng. Life Sci.* 1–6.
- Bouzar, H., Jones, J.B., Stall, R.E., Louws, F.J., Schneider, M., Rademaker, J.L.W., de Bruijn, F.J., Jackson, L.E., 1999. Multiphasic analysis of xanthomonads causing bacterial spot disease on tomato and pepper in the Caribbean and Central America: evidence for common lineages within and between countries. *Phytopathology* 89 (4), 328–335.
- Bradley, D.E., 1967. Ultrastructure of bacteriophages and bacteriocins. *Bacteriol. Rev. Am. Soc. Microbiol.* 31 (4), 230–314. Printed in U.S.A.
- Buonaurio, R., Stravato, V.M., 1992. *Xanthomonas campestris* pv. *vesicatoria* (Doi) Dye su peperone in Italia central. *Inf. Fitopatol.* 42, 49–52.
- Chae, J., Hung, N.B., Yu, S., Lee, H.K., Lee, Y.H., 2014. Diversity of bacteriophages infecting *Xanthomonas oryzae* pv. *oryzae* in paddy fields and its potential to control bacterial leaf blight of rice. *J. Microbiol. Biotechnol.* 24 (6), 740–747.
- Chan, B.K., Abedon, S.T., 2012. Bacteriophage adaptation, with particular attention to issues of phage host range. In: Quiberoni, A., Reinheimer, J. (Eds.), *Bacteriophages in Dairy Processing*. Nova Science Publishers, NY, USA, pp. 25–52.
- Chourasiya, S., Khillare, P.S., Jyethi, D.S., 2015. Health risk assessment of organochlorine pesticide exposure through dietary intake of vegetables grown in the periurban sites of Delhi, India. *Environ. Sci. Pollut. Res. Int.* 22, 5793–5806.
- Civerolo, E.L., 1970. Comparative relationships between two *Xanthomonas pruni* bacteriophages and their bacterial host. *Phytopathology* 60, 1385–1388.
- Civerolo, E.L., Keil, H.L., 1969. Inhibition of bacterial spot of peach foliage by *Xanthomonas pruni* bacteriophage. *Phytopathology* 12, 1966–1967.
- Clark, W.A., 1962. Comparison of several methods for preserving bacteriophages. *Appl. Microbiol.* 10, 466–471.
- EFSA PLH Panel (EFSA Panel on Plant Health), 2014. Scientific Opinion on the pest categorisation of *Xanthomonas campestris* pv. *vesicatoria* (Doi) Dye. *EFSA J.* 12 (6), 3720–3726.
- Gasic, K., Ivanovic, M.M., Calic, A., Obradovic, A., 2011. Isolation and characterization of *Xanthomonas euvesicatoria* bacteriophages. *J. Plant Pathol.* 93 (2), 415–423.
- Gasic, K., Kuzmanovic, N., Ivanovic, M., Prokić, A., Sević, M., Obradović, A., 2018. Complete genome of the *Xanthomonas euvesicatoria* specific bacteriophage K81, its survival and potential in control of pepper bacterial spot. *Front. Microbiol.* 9, 2021.
- Gill, J.J., Hyman, P., 2010. Phage choice, isolation and preparation for phage therapy. *Curr. Pharmaceut. Biotechnol.* 11 (1), 2–14.
- Goodridge, L.D., 2010. Designing phage therapeutics. *Curr. Pharmaceut. Biotechnol.* 11 (1), 15–27.
- Greene, J., Goldberg, R.B., 1985. Isolation and preliminary characterization of lytic and lysogenic phages with wide host range within the streptomycetes. *J. Gen. Microbiol.* 131, 2459–2465.
- Gunnell, D., Eddleston, M., Phillips, M.R., Konradsen, F., 2007. The global distribution of fatal pesticide self-poisoning: systematic review. *BMC Publ. Health* 7, 357.
- Hall, A.R., Scanlan, P.D., Leggett, H.C., Buckling, A., 2012. Multiplicity of infection does not accelerate infectivity evolution of viral parasites in laboratory microcosms. *J. Evol. Biol.* 25, 409–415.
- Hyman, P., 2019. Phages for Phage Therapy: Isolation, Characterization, and Host Range Breadth.
- Iriarte, F., Balogh, B., Momol, M., Smith, L., Wilson, M., Jones, J., 2007. Factors affecting survival of bacteriophage on tomato leaf surfaces. *Appl. Environ. Microbiol.* 73, 1704–1711.
- Jones, J.B., Lacy, G.H., Bouzar, H., Stall, R.E., Schaad, N.W., 2004. Reclassification of the xanthomonads associated with bacterial spot disease of tomato and pepper. *Syst. Appl. Microbiol.* 27, 755–762.
- Jones, J.B., Jackson, L.E., Balogh, B., Obradovic, A., Iriarte, F.B., Momol, M.T., 2007. Bacteriophages for plant disease control. *Annu. Rev. Phytopathol.* 45, 245–262.
- Jones, J.B., Vallad, G.E., Iriarte, F.B., Obradović, A., Wernsing, M.H., Jackson, L.E., Balogh, B., Hong, J.C., Momol, M.T., 2012. Considerations for using bacteriophages for plant disease control. *Bacteriophage* 2 (4), 208–214.
- Jurczak-Kurek, A., Gąsior, T., Nejman-Falenczyk, B., Bloch, S., Dydecka, A., Topka, G., Necel, A., Jakubowska-Deredas, M., Narajczyk, M., Richert, M., Mieszkowska, A., Wróbel, B., Węgrzyn, G., Węgrzyn, A., 2016. Biodiversity of bacteriophages: morphological and biological properties of a large group of phages isolated from urban sewage. *Scientific Reports Sci. Rep.* 6, 34338.
- Kalpage, M.D., De Costa, D.M., 2014. Isolation of bacteriophages and determination of their efficiency in controlling *Ralstonia solanacearum* causing bacterial wilt of tomato. *Trop. Agric. Res.* 26 (1), 140–151.
- Khan, M.M., Nilsson, A.S., 2015. Isolation of phages for phage therapy: a comparison of spot tests and efficiency of plating analyses for determination of host range and efficacy. *PLoS One* 10, e0118557.
- Kizheva, Y., Vancheva, T., Hristova, P., Stoyanova, M., Bogatzevska, N., Moncheva, P., 2011. Diversity of *Xanthomonas* Spp. Causal agents of bacterial spot on pepper and tomato plants in Bulgaria. *Biotechnol. Equip.* 25 (4), 98–104.
- Kizheva, Y., Vancheva, T., Hristova, P., Stoyanova, M., Stojanovska, M., Moncheva, P., Bogatzevska, N., 2013. Identification of *Xanthomonas* strains from tomato and pepper and their sensitivity to antibiotics and copper. *Bulgarian J. Agric. Sci.* 19 (2), 80–82.
- Kizheva, Y., Vancheva, T., Stoyanova, M., Bogatzevska, N., Moncheva, P., Hristova, P., 2016. 16S-23S ITS rDNA PCR-RFLP approach as a tool for identification and differentiation of bacterial spot-causing *Xanthomonas*. *J. Plant Pathol.* 98 (3), 645–649.
- Kizheva, Y., Vancheva-Ebben, T., Hristova, P., Bogatzevska, N., Moncheva, P., 2020. First report of *Xanthomonas euvesicatoria* on tomato in Bulgaria. *Comptes rendus de l'Académie bulgare des Science* 73 (1).
- Kutter, E., 2009. Phage host range and efficiency of plating. *Methods Mol. Biol.* 501, 141–149.
- Liew, K.W., Alvarez, A.M., 1981. Biological and morphological characterization of *Xanthomonas campestris* bacteriophages. *Phytopathology* 71, 269–273.
- Mansfield, J., Genin, S., Magori, S., Citovsky, V., Sriariyanum, M., Ronald, P., Dow, M., Verdier, V., Beer, S.V., Machado, M.A., Toth, I., Salmond, G., Foster, G.D., 2012. Top 10 plant pathogenic bacteria in molecular plant pathology. *Mol. Plant Pathol.* 13 (6), 614–629.
- Marco, G.M., Stall, R.E., 1983. Control of bacterial spot of pepper initiated by strains of *Xanthomonas campestris* pv. *vesicatoria* that differ in sensitivity to copper. *Plant Dis.* 67, 779–781.
- EFSA (European Food Safety Authority), Medina-Pastor, P., Triacchini, G., 2020. The 2018 European Union report on pesticide residues in food. *EFSA J.* 18 (4), 6057–6103.
- Melgarejo, P., Garcia-Jimenez, J., Jordá, M.C., Lopez, M.M., Andrés, M.F., Duran-Vila, N., 2010, second ed.. *Patógenos de plantas descritas en España*. Ministerio de Medio Ambiente y Medio Rural y Marino, Madrid, Spain, pp. 309–310.
- Minsavage, G., Dahlbeck, D., Whalen, M., Kearney, B., Bonas, U., Staskawicz, B., Stal, I.R., 1990. Gene-for-gene relationships specifying disease resistance in *Xanthomonas campestris* pv. *vesicatoria* - pepper interactions. *Mol. Plant Microbe Interact.* 3, 41–47.
- Mitrev, S., Kovačević, B., 2006. Characterisation of *Xanthomonas axonopodis* pv. *vesicatoria* isolated from peppers in Macedonia. *J. Plant Pathol.* 88, 321–324.
- Msimbira, L.A., Jaiswal, S.K., Dakora, F.D., 2016. Identification and characterization of phages parasitic on bradyrhizobia nodulating groundnut (*Arachis hypogaea* L.) in South Africa. *Appl. Soil Ecol.* 108, 334–340.
- Nagy, J.K., Király, L., Schwarczinger, I., 2011. Phage therapy for plant disease control with a focus on fire blight. *Cent. Eur. J. Biol.* 7 (1), 1–12.
- Nicolopoulou-Stamati, P., Maipas, S., Kotampasi, C., Stamatis, P., Hens, L., 2016. Chemical pesticides and human health: the urgent need for a new concept in agriculture. *Front. Public Health* 4, 148.
- Obradović, A., Mavridis, A., Rudolph, K., Janse, J.D., Arsenijević, M., Jones, J.B., Minsavage, G.V., Wang, J.F., 2004. Characterization and PCR-based typing of *Xanthomonas campestris* pv. *tomato* from peppers and tomatoes in Serbia. *Eur. J. Plant Pathol.* 110, 285–292.
- Pernezny, K., Nagata, R., Havranek, N., Sanchez, J., 2008. Comparison of two culture media for determination of the copper resistance of *Xanthomonas* strains and their usefulness for prediction of control with copper bactericides. *Crop Protect.* 27, 256–262.
- Pringsulaka, O., Patarasinpaiboon, N., Suwannasai, N., Athakor, W., Rangsiruji, A., 2011. Isolation and characterisation of a novel *Podoviridae*-phage infecting *Weissella cibaria* N 22 from Nham, a Thai fermented pork sausage. *Food Microbiol.* 28, 518–525.
- Ross, A., Ward, S., Hyman, P., 2016. More is better: selecting for broad host range bacteriophages. *Front. Microbiol.* 7, 1352.
- Sanborn, M., Kerr, K.J., Sanin, L.H., Cole, D.C., Bassil, K.L., Vakil, C., 2007. Non-cancer health effects of pesticides. Systematic review and implications for family doctors. *Can. Fam. Physician* 53, 1712–1720.
- Schwarczinger, I., Nagy, J.K., Kúntler, A., Szabó, L., Geider, K., Király, L., Pogány, M., 2017. Characterization of *Myoviridae* and *Podoviridae* family bacteriophages of *Erwinia amylovora* from Hungary – potential of application in biological control of fire blight. *Eur. J. Plant Pathol.* 149, 639–652.
- Solis-Sánchez, G.A., Quinones-Aguilar, E.E., Fraire-Velázquez, S., Vega-Arreguín, J., Rincón-Enríquez, G., 2020. Complete genome sequence of XaF13, a novel

- bacteriophage of *Xanthomonas vesicatoria* from Mexico. Microbiol. Resour. Announc. 9 e01371-19.
- Suttle, C.A., Chen, F., 1992. Mechanisms and rates of decay of marine viruses in sea water. Appl. Environ. Microbiol. 58, 3721–3729.
- Svircev, A.M., Castle, A.J., Lehman, S.M., 2010. Bacteriophages for control of phytopathogens in food production systems. In: Sabour, P.M., Griffiths, M.W. (Eds.), Bacteriophages in the Control of Food-and Waterborne Pathogens. ASM Press, Washington, DC, pp. 79–96.
- Thayer, P.L., Stall, R.E., 1961. A survey of *Xanthomonas vesicatoria* resistance to streptomycin. Proc. Fla. State Hort. Soc. 75, 163–165.
- Tomlinson, S., 2010. UV Irradiation on Bacteriophage Survival. Honors Theses, p. 142. <https://digitalcommons.coastal.edu/honors-theses/142>.
- Uchiyama, J., Rashel, M., Maeda, Y., Takemura, I., Sugihara, S., Akechi, K., et al., 2008. Isolation and characterization of a novel *Enterococcus faecalis* bacteriophage #EF24C as a therapeutic candidate. FEMS Microbiol. Lett. 278, 200–206.
- Vancheva, T., Stoyanova, M., Tasheva-Terzieva, E., Bogatzevska, N., Moncheva, P., 2018. Molecular methods for diversity assessment among xanthomonads of Bulgarian and Macedonian pepper. Braz. J. Microbiol. 49S, 246–259.
- Vauterin, L., Hoste, B., Kersters, K., Swings, J., 1995. Reclassification of *Xanthomonas*. Int. J. Syst. Bacteriol. 45, 472–489.
- Witczak, A., Abdel-Gawad, H., 2014. Assessment of health risk from organochlorine pesticides residues in high-fat spreadable foods produced in Poland. J. Environ. Sci. Health B 49, 917–928.
- World Health Organization, 1990. Public Health Impact of Pesticides Used in Agriculture. World Health Organization, England.
- Yamada, T., Kawasaki, T., Nagata, S., Fujiwara, A., Usami, S., Fujie, M., 2007. New bacteriophages that infect the phytopathogen *Ralstonia solanacearum*. Microbiology 153, 2630–2639.
- Yu, P., Mathieu, J., Li, M., Dai, Z., Alvarez, P.J., 2016. Isolation of polyvalent bacteriophages by sequential multiple-host approaches. Appl. Environ. Microbiol. 82, 808–815.