ISOLATION AND CHARACTERIZATION OF *XANTHOMONAS EUVESICATORIA* BACTERIOPHAGES

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SUMMARY

Host range, plaque morphology, thermal inactivation point, genome size and restriction fragment patterns of ten bacteriophage isolates originating from soil, pepper seed and irrigation water collected from five localities in Serbia were studied. The bacteriophage isolates were selected based on their specificity to *Xanthomonas euvesicatoria*, causal agent of bacterial spot of pepper. The phages had similar plaque morphology, except for two isolates producing a plaque-surrounding halo in culture of *X. euvesicatoria* strain KFB 189. Four phage isolates were inactivated at 70ºC and six at 71ºC. All phages had genome size of approximately 22 kb and were differentiated into four types by their *Eco*RI and *Bam*HI restriction fragment patterns. Examination of two phages by transmission electron microscopy classified them as A1 morphotype members of the *Myoviridae* family, order *Caudovirales.* Although specific to *X. euvesicatoria*, the phages were differentiated into three groups based on their ability to lyse 59 strains of this bacterium. Adsorption rates and one-step growth curves were determined for each group representative phage isolates.

Key words: phage host range, restriction analysis, electron microscopy.

INTRODUCTION

Bacterial spot, caused by *Xanthomonas euvesicatoria* (formerly *X. campestris* pv. *vesicatoria* group A, Jones *et al.*, 2004), is an economically very important disease of pepper in Serbia (Balaz, 1994; Obradović *et al.*, 1999, 2000, 2001). Routine disease management practices, such as use of good quality seed, crop rotation, growth of less susceptible cultivars and application of copper compounds, have failed to provide satisfactory disease control, especially when weather conditions favored the spread of the pathogen. Therefore, new approaches are needed for effective control of *X. euvesicatoria* infections.

From the period of their discovery, in the early twentieth century, the possibility of using bacteriophages for the control of various bacterial diseases, including those on plants, has been extensively studied. Despite the promising early results, the use of antibiotics and copper compounds became the standard for preventing and controlling phytopathogenic bacteria. Due to occurrence of antibiotic-resistant and copper-tolerant or resistant strains (Marco and Stall, 1983; Minsavage *et al.*, 1990; Thayer and Stall, 1961), interest in phage therapy has increased in the recent years. Being simple for application, suitable for combination with other plant protection treatments and cost effective, bacteriophages have been used as a part of integrated disease management in several pathosystems (Lang *et al.*, 2007; Obradović *et al.*, 2004a, 2005; Svircev *et al.*, 2006; Tanaka *et al.*, 1990). Moreover, because of their host specificity, phages can be effectively used for the control of pathogenic bacteria without affecting other members of the bacterial community (Gill and Abedon, 2003).

A number of *Xanthomonas* spp. specific bacteriophages have been tested for plant disease control, i.e. bacterial spot of *Prunus* spp., leaf blight of rice, tomato leaf spot, bacterial blight of geranium and onion, citrus canker and bacterial spot (Jones *et al.*, 2007). Thus, the biological and morphological properties of some filamentous and tailed *X. campestris* phages have been studied (Liew and Alvarez, 1981; Lin *et al.*, 1994; Suton *et al.*, 1958; Tseng *et al.*, 1990; Watanabe *et al.*, 1980), but not those of phages infecting *X. euvesicatoria*.

The objective of this study was to determine occurrence of *X. euvesicatoria*-specific bacteriophages by isolating them from various substrates collected from pepper-growing areas of Serbia, and to characterize them by studying their host range, phage virion and plaque morphology, thermal inactivation, genome size and restriction fragment length polymorphism (RFLP).

MATERIAL AND METHODS

Bacterial strains and media. Bacterial strains used in this study included *X. vesicatoria* NCPPB 1423 and

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KFB 29 (Obradović *et al*., 2004b), *X. perforans* NCPPB 4321 and 91-118 RIF (Tudor-Nelson *et al.*, 2003) and *X. gardneri* NCPPB 881 and ATCC 19865. These strains were used to test phage host range within pepper and tomato-associated xanthomonads. A further group of strains of *X. euvesicatoria* isolated from diseased pepper plants originating from 26 locations in Serbia during 1996, 1997 and 2008 (Ignjatov *et al*., 2010; Obradović *et al*., 2004b) was used for testing phage specificity for the strains of *X. euvesicatoria* population present in Serbia. All strains were routinely maintained in nutrient broth (NB) supplemented with 30% glycerol at -80°C. During the experiments bacterial strains were subcultured on nutrient agar plates (NA) incubated at 27°C. For bacteriophage detection and propagation either semisolid nutrient agar yeast extract medium, NYA, (0.8% Nutrient Broth, 0.6% Bacto Agar and 0.2% Yeast Extract) or NB were used.

Isolation and purification of phages. Isolation of bacteriophages was attempted from the following substrates: above-ground parts (stems, leaves and fruits) of pepper plants, pepper rhizosphere soil, pepper seeds and irrigation water, collected from 15 localities in Serbia during 2005, 2007 and 2008. To increase the potential of *X. euvesicatoria*-specific phage isolation, substrate samples were subjected to incubation with target bacteria in 50 ml NB buffered with 2.5 g $CaCO_3$. Flasks with medium were inoculated with 5 ml water-suspension of either *X. euvesicatoria* strain KFB 1 or KFB 13 (10^8 CFU ml^{-1}, OD_{600} = 0.3) from a 24 h NA culture. To enrich phage populations, samples of either irrigation water (50 ml), plant tissue (5 g) or soil (10 g) were added to bacterial liquid cultures and incubated on a rotary shaker for 24 h at 27°C. Aliquots (1 ml) of the enrichment culture were centrifuged at 16,000 *g* for 5 min to remove cells and debris. The supernatant was treated with chloroform (10% v/v) for 20 min and resulting suspensions were stored in microfuge tubes at 4°C.

All suspensions were tested for the presence of virulent phages by screening for lysis of the target bacterium previously used for the enrichment. A 100 µl of bacterial suspension (10^8 CFU ml^{-1}) in sterile tap water from a 24-h-old NA culture, was pipetted in the center of a sterile Petri dish (90 mm in diameter). At the same time, NYA medium was autoclaved, cooled to 48°C in a water-bath then poured into plates (16 ml/plate) and mixed with bacterial suspension by swirling motion (pour-plate procedure). When the medium solidified, duplicate plates were spot-inoculated by pipetting 10 µl of the testing phage suspensions onto the medium surface, and positive reactions were scored as either clear or turbid plaques or zones of confluent lysis within the inoculated area after 24-48 h incubation.

Phage purification was done by three subsequent single plaque isolation steps. From the plaques in the isolation plates, phages were transferred by stabbing the particular plaque with a sterile tooth pick and dipping the tip into 100 µl of sterile tap water in a microfuge tube. Two 10-fold dilutions of this suspension were prepared and 100 µl of each was pipette-mixed with 100 µl of the host bacterium suspension at the bottom of the Petri dish, followed by pour-plate procedure. The inoculated media were incubated at 27°C for 24 h. After a third purification step, phages were extracted by adding 5 ml of sterile tap water onto the plate, crushing the medium, and centrifuging at 8,000 *g* for 20 min. The supernatant was transferred to a microfuge tube, treated with chloroform (10% v/v) for 20 min and these purified phage suspensions were stored at 4°C for further testing.

Phage titer was determined by plating 100 µl of 10-fold dilutions of purified phage suspensions and 100 µl of the bacterial suspension in NYA medium as described above. After 24 h incubation, phage concentration was estimated according to the formula for bacterial enumeration (Klement *et al.*, 1990) and was expressed as "plaque forming units per ml" (PFU ml^{-1}).

Optimal multiplicity of infection. Multiplicity of infection (MOI) is defined as the ratio between virus particles and host cells (Birge, 2000). To determine optimal MOI, *X. euvesicatoria* strain KFB 189 was grown in NB at 27°C until it reached approximately 10^8 CFU ml^{-1} (OD_{600} = 0.3). Bacterial concentration was also checked by counting CFUs obtained by dilution plating on NA medium (Klement *et al.*, 1990). These early log phase cells were infected with KΦ 1 phage at four different ratios (approx. 0.01, 0.1, 1, and 10 PFU/CFU). After 18 h incubation on a rotary shaker at 150 rpm at 27°C, cultures were chloroform treated (10% v/v) and assayed to determine phage titer. Assays were performed in triplicate. MOI resulting in highest phage titer within 18 h incubation was considered as an optimal MOI and used in subsequent phage propagation.

Propagation and preservation of phages. Propagation of phage isolates was done by infecting actively

Table 1. List of *X. euvesicatoria* specific bacteriophages isolated in Serbia and studied in this paper.

Phage isolate	Source	Locality	Year of isolation
KΦ 1	Soil	Druzetic	2005
KΦ 2	Pepper seed	Medveda	2007
KΦ 3	Soil	Medveda	2007
KΦ 4	Irrigation water	Medveda	2007
KΦ 5	Soil	Brus	2007
KΦ 6	Pepper seed	Medveda	2007
KΦ 7	Soil	Medveda	2007
KΦ 8	Soil	Brus	2007
KΦ 9	Soil	Despotovo	2008
KΦ 15	Soil	Tovarisevo	2008

growing culture (*ca.* 10^8 CFU ml^{-1}) of *X. euvesicatoria* strain KFB 189 in NB medium at MOI of 0.1. After 24 h incubation on a rotary shaker at 150 rpm and 27°C, the culture was chloroform treated (10% v/v) and stored at 4°C. For long-term storage, 100 µl of high titer phage suspension and 100 µl of bacterial suspension were pipette-mixed and left 5 min to facilitate phage adsorption followed by transfer to 30% glycerol NB and storing at -80°C.

Host range analysis. Host range of the phage isolates was determined by studying their lytic activity in cultures of pepper- and tomato-associated xanthomonads (*X. euvesicatoria*, *X. vesicatoria*, *X. perforans* and *X. gardneri*) (Table 2). Further variation of specificity was checked by infecting 59 strains of *X. euvesicatoria* isolated from pepper in Serbia. Bacterial lawn was prepared by suspending 24-h-old bacteria in sterile tap water (10^8 CFU ml^{-1}) followed by pipetting 100 µl of this suspension at the bottom of an empty Petri dish subjected to the pour-plate procedure. When the medium solidified, 4 µl of each phage suspension was spotted in a particular order onto its surface. Plates were kept in a laminar flow hood for 30 min allowing phage suspension drops to diffuse into the medium and incubated at 27°C for 24-48 h. Phage activity was scored on the basis of plaque formation indicating host cell lysis. The plaques were categorized as clear or turbid. The experiment was performed in triplicate.

Thermal inactivation. Heat stability of phage isolates was studied by exposing 1 ml of phage suspension in sterile tap water (10^7 PFU ml^{-1}) to temperatures from 35 to 75°C at 5°C interval in a water bath. To determine the thermal inactivation point more accurately the experiment was further repeated three times at 1°C interval for temperatures ranging from 65 to 75°C. After 10 min incubation, the phage suspensions in glass tubes were rapidly chilled in crushed ice before being assayed by spotting 5 µl on *X. euvesicatoria* lawn (KFB 189). Formation of plaques, as a sign of phage activity, was recorded after 24 h incubation at 27°C.

Phage adsorption. To determine the number of phage particles adsorbed to host cells within a particular period, an adsorption experiment was carried out as described by Ellis and Delbrück (1939). Three phage isolates, KΦ 1, KΦ 8 and KΦ 15 differing in specificity to *X. euvesicatoria* strains, were used for adsorption and one-step growth kinetics study. One milliliter of actively growing *X. euvesicatoria* strain KFB 189 (10^8 CFU ml^{-1}) in NB was infected by phage suspension to reach MOI of 0.1, and incubated at 27°C. At predetermined intervals (1, 3, 5, 10 and 20 min) mixtures were centrifuged at 10,000 *g* for 5 min to sediment the phage-adsorbed bacteria. Titers of unadsorbed free phages in the supernatant were determined as described above (see "Isolation and purification of phages"), and the results were expressed as percentages of the initial phage counts. The experiment was repeated three times.

One-step growth. Phage life cycle was studied by a one-step growth procedure and the number of particles liberated from one cell was expressed as "burst size". A modification of the protocols of Ellis and Delbrück (1939) and Carlson (2005) was used. One milliliter of exponential-growth-phase culture of *X. euvesicatoria* (KFB 189) in NB (10^8 CFU ml^{-1}) and phage suspension were mixed at MOI of 0.1. The mixture was incubated at 27°C for 5 min to allow phage adsorption. Immediately thereafter, the mixture was diluted to 10^{-4} obtaining in total 20 ml of NB in 50 ml Erlenmeyer flasks. The mixture dilution minimizes the chance of unadsorbed or released phages to infect new bacterial cells and therefore increase in phage concentration represented the number of newly multiplied phage particles. Diluted mixture was incubated at 27°C in a water bath and 100 µl samples were subsequently taken at 10 min intervals. Phage titers were monitored on NYA plates, as described above. The experiment was performed three times. Latent period was defined as the time interval between the adsorption (not including 5 min pre-incubation) and the beginning of the first burst indicated by the initial rise in phage titer (Adams, 1959; Ellis and Delbrück, 1939). Burst size was calculated as the ratio between the final count of liberated phage particles and the initial count of infected bacterial cells during the latent period (Adams, 1959).

DNA extraction. Bacteriophage DNA was extracted according to Balogh (2006). Purified phage suspensions (1.5 ml, 10^{10} PFU ml^{-1}) in SM buffer [0.05 M Tris-HCl pH 7.5, 0.1 M NaCl, 10 mM $MgSO_4$ and 1% (w/v) gelatin] were treated with 12.3 µl/sample of DNase I (1 unit/µl) and 90 µg/sample of RNase A (10 µg/µl) (MBI Fermentas, Lithuania) for 30 min at 37°C. Subsequently, samples were divided into 500 µl subsamples, and mixed with 375 µl of a phenol-chloroform-isoamyl alcohol mixture (25:24:1). After centrifugation for 5 min at 10,000 *g*, the top aqueous layer was transferred into a new microfuge tube. The sample volume was brought up to 500 µl with sterile deionized (DI) water and subjected to phenol-chloroform-isoamyl alcohol extraction two more times. Afterwards, sterilized DI water was added to the sample to bring the volume to 500 µl. The solution was treated with 250 µl of chloroform-isoamyl alcohol (24:1), vortexed and centrifuged for 5 min at 10,000 *g*. The top aqueous phase containing DNA was saved and transferred to a new microtube. The DNA was precipitated by adding 40 µl sodium acetate (3 M, pH 5.2) and 800 µl cold (-4°C) 95% ethanol, vortexed and incubated for 30 min at -80°C. The sample was

Table 2. Bacteriophage specificity to xanthomonads associated with pepper and tomato.

Bacterial strain	*Xanthomonas* sp.	Locality of isolation	Bacteriophage isolate									
			KΦ 1	KΦ 2	KΦ 3	KΦ 4	KΦ 5	KΦ 6	KΦ 7	KΦ 8	KΦ 9	KΦ 15
KFB 29	*X. vesicatoria*	Lozovik	–	–	–	–	–	–	–	–	–	–
KFB 0108	*X. vesicatoria*	NCPPB 1423	–	–	–	–	–	–	–	–	–	–
KFB 061	*X. perforans*	91-118 RIF	–	–	–	–	–	–	–	–	–	–
KFB 0109	*X. perforans*	NCPPB 4321	–	–	–	–	–	–	–	–	–	–
KFB 0111	*X. gardneri*	NCPPB 881	–	–	–	–	–	–	–	–	–	–
KFB 0116	*X. gardneri*	ATCC 19865	–	–	–	–	–	–	–	–	–	–
RKFB 112	*X. euvesicatoria*	Horgoš	+	+	+	+	+	+	+	+	+	+
RKFB 113	*X. euvesicatoria*	Horgoš	+	+	+	+	+	+	+	+	+	+
RKFB 114	*X. euvesicatoria*	Horgoš	+	+	+	+	+	+	+	+	+	+
RKFB 115	*X. euvesicatoria*	Horgoš	+	+	+	+	+	+	+	+	+	+
RKFB 116	*X. euvesicatoria*	Horgoš	+	+	+	+	+	+	+	+	+	+
RKFB 164	*X. euvesicatoria*	Kula	(+)	(+)	(+)	(+)	(+)	(+)	+	+	+	–
RKFB 165	*X. euvesicatoria*	Topola	(+)	(+)	(+)	(+)	(+)	(+)	+	+	+	–
RKFB 167	*X. euvesicatoria*	Kula	+	+	+	+	+	+	+	+	+	+
RKFB 189	*X. euvesicatoria*	Kula	+	+	+	+	+	+	+	+	+	+
RKFB 191	*X. euvesicatoria*	Despotovo	(+)	(+)	(+)	(+)	(+)	(+)	+	+	+	–
RKFB 192	*X. euvesicatoria*	Despotovo	(+)	(+)	(+)	(+)	(+)	(+)	+	+	+	–
RKFB 198	*X. euvesicatoria*	Despotovo	+	+	+	+	+	+	+	+	+	+
RKFB 202	*X. euvesicatoria*	Horgoš	+	+	+	+	+	+	+	+	+	+
RKFB 203	*X. euvesicatoria*	Horgoš	+	+	+	+	+	+	+	+	+	+
RKFB 204	*X. euvesicatoria*	Horgoš	+	+	+	+	+	+	+	+	+	+
RKFB 205	*X. euvesicatoria*	Horgoš	+	+	+	+	+	+	+	+	+	+
RKFB 208	*X. euvesicatoria*	Smederevo	(+)	(+)	(+)	(+)	(+)	(+)	+	+	+	–
RKFB 212	*X. euvesicatoria*	Smederevo	+	+	+	+	+	+	+	+	+	+
RKFB 213	*X. euvesicatoria*	Smederevo	+	+	+	+	+	+	+	+	+	+
RKFB 216	*X. euvesicatoria*	Bačka Palanka	+	+	+	+	+	+	+	+	+	+
RKFB 217	*X. euvesicatoria*	Bačka Palanka	+	+	+	+	+	+	+	+	+	+
RKFB 218	*X. euvesicatoria*	Tovariševo	(+)	(+)	(+)	(+)	(+)	(+)	+	+	+	–
RKFB 220	*X. euvesicatoria*	Bašaid	+	+	+	+	+	+	+	+	+	+
RKFB 221	*X. euvesicatoria*	Bašaid	+	+	+	+	+	+	+	+	+	+
RKFB 223	*X. euvesicatoria*	Senta	+	+	+	+	+	+	+	+	+	+
RKFB 224	*X. euvesicatoria*	Senta	+	+	+	+	+	+	+	+	+	+
RKFB 227	*X. euvesicatoria*	Kikinda	(+)	(+)	(+)	(+)	(+)	(+)	+	+	+	–
RKFB 228	*X. euvesicatoria*	Kikinda	(+)	(+)	(+)	(+)	(+)	(+)	+	+	+	–
RKFB 231	*X. euvesicatoria*	Novi Kneževac	+	+	+	+	+	+	+	+	+	+
RKFB 235	*X. euvesicatoria*	Novi Kneževac	(+)	(+)	(+)	(+)	(+)	(+)	+	+	+	–
RKFB 236	*X. euvesicatoria*	Novi Kneževac	(+)	(+)	(+)	(+)	(+)	(+)	+	+	+	–
RKFB 239	*X. euvesicatoria*	Gospođinci	+	+	+	+	+	+	+	+	+	+
RKFB 240	*X. euvesicatoria*	Gospođinci	+	+	+	+	+	+	+	+	+	+
RKFB 241	*X. euvesicatoria*	Gospođinci	+	+	+	+	+	+	+	+	+	+
RKFB 243	*X. euvesicatoria*	Gložan	+	+	+	+	+	+	+	+	+	+
RKFB 244	*X. euvesicatoria*	Gložan	+	+	+	+	+	+	+	+	+	+
RKFB 248	*X. euvesicatoria*	Gložan	+	+	+	+	+	+	+	+	+	+
RKFB 249	*X. euvesicatoria*	Gložan	+	+	+	+	+	+	+	+	+	+
RKFB 251	*X. euvesicatoria*	Pivnice	+	+	+	+	+	+	+	+	+	+
RKFB 252	*X. euvesicatoria*	Pivnice	+	+	+	+	+	+	+	+	+	+
RKFB 255	*X. euvesicatoria*	Silbaš	(+)	(+)	(+)	(+)	(+)	(+)	+	+	+	–
RKFB 257	*X. euvesicatoria*	Vukovar	+	+	+	+	+	+	+	+	+	+
RKFB 261	*X. euvesicatoria*	Ruski Krstur	+	+	+	+	+	+	+	+	+	+
RKFB 262	*X. euvesicatoria*	Ruski Krstur	+	+	+	+	+	+	+	+	+	+
RKFB 265	*X. euvesicatoria*	Kula	+	+	+	+	+	+	+	+	+	+
RKFB 266	*X. euvesicatoria*	Kula	+	+	+	+	+	+	+	+	+	+
RKFB 267	*X. euvesicatoria*	Odžaci	+	+	+	+	+	+	+	+	+	+
RKFB 269	*X. euvesicatoria*	Vrbas	(+)	(+)	(+)	(+)	(+)	(+)	+	+	+	–
RKFB 271	*X. euvesicatoria*	Kucura	+	+	+	+	+	+	+	+	+	+
RKFB 274	*X. euvesicatoria*	Stanišić	+	+	+	+	+	+	+	+	+	+
RKFB 275	*X. euvesicatoria*	Stanišić	+	+	+	+	+	+	+	+	+	+
RKFB 276	*X. euvesicatoria*	Lalić	+	+	+	+	+	+	+	+	+	+
RKFB 279	*X. euvesicatoria*	Lalić	+	+	+	+	+	+	+	+	+	+
RKFB 282	*X. euvesicatoria*	Sombor	+	+	+	+	+	+	+	+	+	+
RKFB 283	*X. euvesicatoria*	Sombor	+	+	+	+	+	+	+	+	+	+
RKFB 284	*X. euvesicatoria*	Ratkovo	+	+	+	+	+	+	+	+	+	+
KFB 189	*X. euvesicatoria*	Družetić	+	+	+	+	+	+	+	+	+	+
KFB 1	*X. euvesicatoria*	Kovilj	+	+	+	+	+	+	+	+	+	+
KFB 13	*X. euvesicatoria*	Horgoš	+	+	+	+	+	+	+	+	+	+

+ clear plaque formation, (+) turbid plaque formation, - no plaque formation.

then centrifuged (10,000 *g* for 20 min at 4°C), the supernatant was discarded and 1 ml of 70% ethanol was added. After centrifugation (10,000 *g* for 5 min) the supernatant was gently removed and the pellet containing the precipitated DNA was allowed to air dry. The pellet was resuspended in 10 µl sterile DI water and stored at 4°C for further analysis.

Restriction analysis of phage DNA. Bacteriophage DNA was digested with *Bam*HI and *Eco*RI restriction enzymes according to the supplier's instructions (MBI Fermentas, Lithuania). Three microliters of the phage DNA suspension were mixed with 5 µl sterile DI water, 1 µl enzyme buffer and 1 µl restriction enzyme (10 units/µl) (*Eco*RI or *Bam*HI). The mixture was incubated at 37°C for 90 min. DNA fragments were separated by agarose (1%) gel electrophoresis in Tris-acetate-EDTA buffer, stained in ethidium bromide (1 µg ml^{-1}) and visualized by a digital imaging camera (Vilber Lourmat, France).

Transmission electron microscopy. The morphology of two phage isolates KΦ 1 and KΦ 15, differing in specificity to different *X. euvesicatoria* strains, was examined by transmission electron microscope (TEM - Philips CM12) according to the negative staining protocol described by Gill *et al.* (2003) and Balogh (2006). Bacteriophage suspension (10^{10} PFU/ml) was centrifuged at 16,000 *g* for 1 h at 5°C. Supernatant was discarded and the phage pellet was resuspended in 100 µl of sterile distillated water. A drop of the phage suspension was applied to 400 mesh formvar-coated copper grid and allowed to sit for 2 min. The liquid was blotted away and the grid was rinsed with DI water. A 1% uranyl acetate solution was applied to the grid and blotted away after 1 min. The phages were observed and photographed by TEM with an accelerating voltage of 80 kV. Average phage dimensions were calculated by measuring the head diameter and tail length of ten phage particles for each isolate.

RESULTS

Phage isolation. A total of 25 bacteriophages were isolated during 2005, 2007 and 2008, 22 phages from rhizosphere soil, two from pepper seed and one from irrigation water. Phage isolation from pepper stems, leaves and fruits was unsuccessful. Purification and propagation procedures resulted in high titer phage stock suspensions, containing 10^{10} PFU ml^{-1}. Ten phage isolates (KΦ 1-9 and KΦ 15) were selected for further characterization based on their substrate origin and host specificity (Table 1). The isolates formed clear plaques *ca.* 3 to 4 mm in diameter, with sharp edges, on lawns of *X. euvesicatoria* strain KFB 189 after 24 h incubation. Phage isolates KΦ 7 and KΦ 9 produced plaques of the same size with an additional translucent halo which continued to expand after the plaque itself stopped growing. The optimal MOI of KΦ 1 phage was determined to be 0.10 - 0.15.

Host range. All tested phage isolates were specific to *X. euvesicatoria* only. They did not lyse any of the *X. vesicatoria*, *X. gardneri* or *X. perforans* strains. The results of the spot test showed that phages could be divided into three groups based on their specificity to 59 *X. euvesicatoria* strains. Except phage KΦ 15, all isolates formed plaques on all of the 59 *X. euvesicatoria* strains tested (Table 2). Clear plaques on the lawn of *X. euvesicatoria* strains indicated complete cell lysis and turbid plaques partial lysis. Phage isolates KΦ 7, KΦ 8 and KΦ 9 formed clear plaques on all *X. euvesicatoria* strains and were placed into group I. Group II consisted of phages KΦ 1, KΦ 2, KΦ 3, KΦ 4, KΦ 5 and KΦ 6, which formed turbid plaques on 12 strains and clear plaques on the remaining 47 strains of *X. euvesicatoria*. Phage isolate KΦ 15 belonged to group III, since it lysed only 47 *X. euvesicatoria* strains, showing the narrowest host range.

Thermal inactivation. Phages KΦ 2, KΦ 6, KΦ 8 and KΦ 9 were inactivated after 10 min of exposure to 70°C, while phage isolates KΦ 1, KΦ 3, KΦ 4, KΦ 5, KΦ 7 and KΦ 15 were inactivated at 71°C.

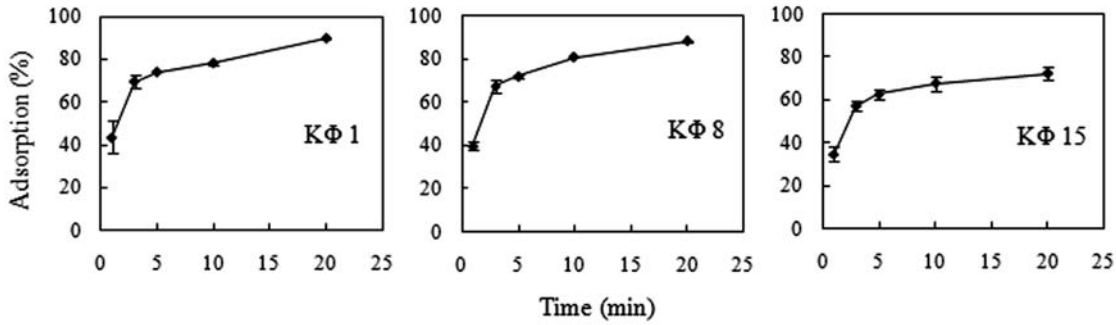

Fig. 1. Adsorption curve of bacteriophages KΦ 1, KΦ 8 and KΦ 15 to *X. euvesicatoria* KFB 189 cells. Means ± standard error from three independent experiments are shown. Some of the error bars were too small to be included.

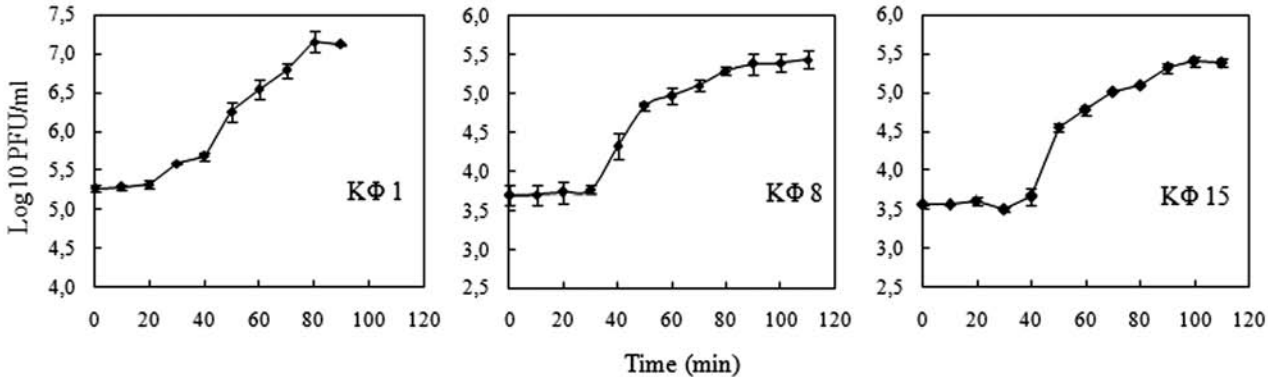

Fig. 2. One-step growth curve for bacteriophage KΦ 1, KΦ 8 and KΦ 15 propagated in *X. euvesicatoria* KFB 189. Means ± standard error from three independent experiments are shown. Some of the error bars were too small to be included.

Adsorption studies and one-step growth. Adsorption of phages KΦ 1, KΦ 8 and KΦ 15 to *X. euvesicatoria* (KFB 189) cells in NB medium at 27°C after 20 min incubation was 90, 88 and 72.4%, respectively (Fig. 1). The proliferation rate of bacteriophages, determined by the latent period and the burst size, were calculated from the one-step growth curve (Fig. 2). Phage KΦ 1 had latent period of 20 min and burst size of 75±4 plaque forming units per infected cell. However, phages KΦ 8 and KΦ 15 had latent period 30 min and burst size of 74±22 and 70±11 PFU per infected cell, respectively. The rise period i.e. lysis of simultaneously infected bacterial cells during which the phage population continually increased, lasted *ca.* 60 min for phage isolates KΦ 1 and KΦ 8 and 70 min for phage KΦ 15.

Analysis of phage DNA. Genomic DNA of ten phage isolates was analyzed by restriction enzyme digestion and agarose gel electrophoresis. All phage isolates had similar genome size of approximately 22 kb (Fig. 3a). Based on restriction analysis with *Bam*HI and *Eco*RI (Fig. 3b,c), phages were divided into four RFLP groups (A, B, C, D). Isolates KΦ 1, KΦ 2, KΦ 3, KΦ 4 and KΦ 5 had identical restriction patterns in both digestion procedures, showing high DNA homology, and formed group A. Another high DNA homology group consisted of isolates KΦ 8 and KΦ 9 and was designated as B. Isolates KΦ 6 and KΦ 15 had an identical *Eco*RI pattern with the A group, but their *Bam*HI pattern was identical to that of the B group, thus were placed into new group C. Unlike group C isolates, isolate KΦ 7 had an identical *Eco*RI pattern with group B but a *Bam*HI pattern identical to group A and represented the forth group D.

Transmission electron microscopy. Electron micrographs revealed that phages KΦ 1 and KΦ 15 had particles of similar size and belonged to the order *Caudovirales*, family *Myoviride* based on the presence of an icosahedral head, a neck/collar region and a sheathed

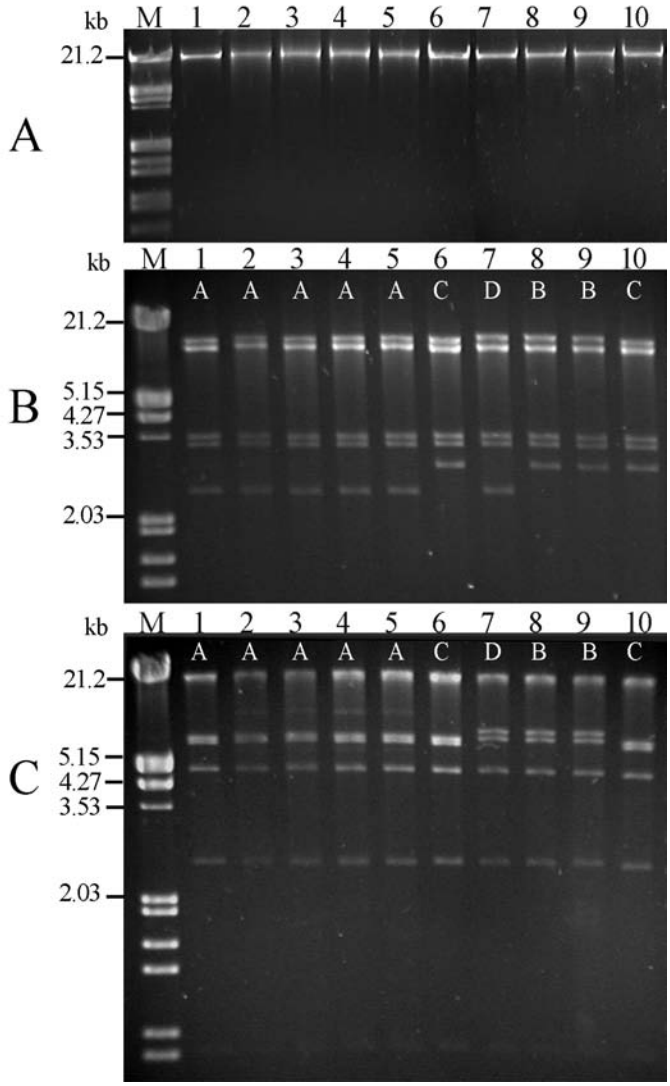

Fig. 3. Agarose gel electrophoresis of DNAs from *X. euvesicatoria* specific bacteriophages. Undigested bacteriophages' DNA (A); bacteriophage DNA digested with *Bam*HI (B) and *Eco*RI (C) enzymes. Lane 1 - 9: KΦ 1 - 9; lane 10: KΦ 15; M - Marker (Lambda DNA/*Eco*RI + *Hin*DIII Marker, Fermentas, Lithuania).

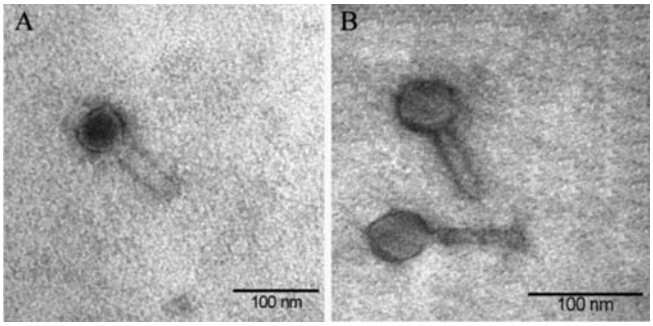

Fig. 4. Electron micrographs of *X. euvesicatoria* specific bacteriophages. Bacteriophage isolate KΦ 1 (A) and KΦ 15 (B).

rigid tail (Fig. 4). Heads of KΦ 1 and KΦ 15 isolates were 51.8±2.5 nm and 55.9±2.3 nm in diameter (means ± standard deviations), respectively. The length of tails was 79.0±9.0 nm for phage KΦ 1 and 80.0±3.3 nm for phage KΦ 15.

DISCUSSION

Use of bacteriophages for control of plant bacterial diseases is a fast developing research field with great potential (Balogh *et al*., 2003, 2009; Jones *et al.*, 2007). Public concerns about environment pollution by pesticides and increasing popularity of organic production has contributed to the growth of biological control agent studies and their use in plant disease management. However, the efficient use of biocontrol agents requires good understanding of their biological characteristics and efficacy.

In this study, we isolated 25 *X. euvesicatoria*-specific bacteriophages from various samples collected from different locations in Serbia. The majority of the phages came from the soil of pepper fields. Phage isolation from pepper phyllosphere was not successful. Difficulties in phage isolation from phyllosphere have already been reported (Erskine, 1973; Flaherty *et al*., 2001; Gill *et al*., 2003; Okabe and Goto, 1963), as it is a hostile environment where phages degrade extremely rapidly due to desiccation or UV light. Poor survival of bacteriophages on plant leaf surfaces is a major limiting factor of phage treatments. Although soil provides protection from desiccation and UV light (Erskine, 1973; Iriarte *et al*., 2007), several factors, such as phage diffusion through the heterogeneous soil matrix, biofilms or soil particles trapping phages irreversibly, low soil pH and physical obstacles, can limit phage efficiency in controlling disease in the rhizosphere (Gill and Abedon, 2003; Reanney *et al*., 1983; Williams *et al.*, 1987). Moreover, the use of chloroform during isolation may have contributed to the loss of some isolates due to phage sensitivity to this compound (Ackermann and DuBow, 1987). Phages that are sensitive to the above mentioned environmental stress factors and populations hard to culture or maintain, are not particularly desirable as potential biological control agents.

Out of 25, we selected ten phage isolates based on their source of isolation and host specificity. They were further characterized by studying their plaque morphology, host range, thermal inactivation, genome size and restriction fragment patterns. All phage isolates formed plaques of the same morphology with exception of two isolates forming an additional halo surrounding the plaques. The presence of a halo could be an indication of soluble enzymes degrading extracellular polymeric structures such as exopolysaccharides from the host strain (Abedon and Yin, 2008). Production of a halo by *Erwinia amylovora*-specific phages was shown to result from production of a polysaccharide depolymerase, capable of hydrolyzing the capsular polysaccharide (Hartung *et al*., 1988; Ritchie and Klos, 1977; Schnabel and Jones, 2001).

Control of plant pathogenic bacteria using virulent phages with a narrow host range restricted to certain strains of the target only, largely restrain practical applications (Okabe and Goto, 1963; Vidaver, 1976). However, high host specificity minimizes the probability for unwanted phage attack to beneficial bacteria. Host range studies showed that among all xanthomonads affecting pepper and tomato, our phage isolates were specific just to *X. euvesicatoria*, but they were able to infect a wide range of target bacterial strains. Phage isolates KΦ 7, KΦ 8 and KΦ 9 effectively lysed all the *X. euvesicatoria* strains, and therefore could be used as an additional treatment for controlling pepper bacterial spot. Application of more than one isolate mixture avoids potential problems with host resistance (Schnabel *et al*., 1999). In order to prevent occurrence of phage-resistant mutants, Jackson (1989) developed a strategy that involved preparing mixtures of host range mutant phages (h-mutants) for disease control. H-mutants possess the ability to lyse bacterial strains resistant to the parent phage (Adams, 1959), while maintaining the ability to lyse the wild type bacterium.

A one-step growth experiment showed that phages KΦ 1, KΦ 8 and KΦ 15 had typical growth cycle and burst size of 75±4, 74±22 and 70±11 PFU per infected cell, respectively. These burst sizes are within the range of 50 to 100 PFU for tailed phages of enteric Gram negative bacteria and pseudomonads (Ackermann and DuBow, 1987). We found that all phages were inactivated by temperatures of 70°C and above. Similar results were obtained by Liew and Alvarez (1981) for a *X. campestris* phage of the *Myoviridae* family and by Civerolo (1970) for *X. pruni* phages.

Like most tailed phages, our isolates have a genome consisting of a linear, double-stranded DNA. The estimated genome size (*ca.* 22 kb) is characteristic for all isolates, and is smaller than those of other known

phages from family *Myoviridae.* However, a similar genome size (about 21 kb) was reported for a *Bacillus subtilis* phage belonging to the same family (Kim *et al.*, 2011). The use of restriction endonucleases provided phage differentiation into four groups. We did not observe any correlation between RFLP groups, location or substrates the phages were isolated from, or the phage host range.

The two phages studied by TEM had the same morphology. Based on the classification system described by Bradley (1967) and improved by Ackermann and DuBow (1987), these phages fit into the order *Caudovirales*. The aspect of phage head and tail (Fig. 4) indicate that they belong to the family *Myoviridae* which contains phages with contractile tails. This morphotype, however, is characteristic for the A1 type described by Ackermann and DuBow (1987). In an extensive review of morphology of phage particles carried out by Ackermann (2007), the genus *Xanthomonas* has at least 35 types of tailed phages, 25 (71%), 9 (26%) and 1 (3%) of which belong in the family *Siphoviridae*, *Myoviridae* and *Podoviridae,* respectively.

The phage isolates and their characteristics described in this paper provide a solid base for further study of their ecology and application efficacy, in view of the inclusion of bacteriophage treatment in pepper bacterial spot management practices. This treatment is supposed to contribute to an improved success of such an integrated bacterial spot control strategy and to reduce the use of conventional pesticides, which is beneficial to the environment and human and animal health.

ACKNOWLEDGEMENTS

This research was supported by the Ministry of Science and Technological Development, Republic of Serbia, project: III46008: *Development of integrated management of harmful organisms in plant production in order to overcome resistance and to improve food quality and safety*. We thank Dr. Aleksandra Korać and her coworkers from the Institute of Zoology, Faculty of Biology, University of Belgrade, for electron microscopy analysis, and Dr. Jaap D. Janse for critical review of the manuscript.

REFERENCES

Abedon S.T., Yin J., 2008. Impact of spatial structure on phage population growth. In: Abedon S.T. (ed.). Bacteriophage Ecology, Population Growth, Evolution, and Impact of Bacterial Viruses, pp. 94-113. Cambridge University Press, Cambridge, UK.

Ackermann H.W., 2007. 5500 Phages examined in the electron microscope. *Archives of Virology* **152**: 227-243.

Ackermann H.W., DuBow M.S., 1987. General properties of bacteriophages. In: Viruses of Prokaryotes, vol I. CRC Press, Boca Raton, Florida, USA.

Adams M.H., 1959. Bacteriophages. Interscience Publishers, New York, NY, USA.

Balaz J., 1994. Leaf spot of pepper caused by bacterium *Xanthomonas campestris* pv. *vesicatoria*. *Contemporary Agriculture* **42**: 341-345.

Balogh B., Jones J.B., Momol M.T., Olson S.M., Obradović A., King B., Jackson L.E., 2003. Improved efficacy of newly formulated bacteriophages for management of bacterial spot of tomato. *Plant Disease* **87**: 949-954.

Balogh B., 2006. Characterization and use of bacteriophages associated with citrus bacterial pathogens for disease control. Ph.D. Thesis. University of Florida, USA.

Balogh B., Momol M.T., Obradović A., Jones J.B., 2009. Bacteriophages as agents for the control of plant pathogenic bacteria. In: Walters D. (ed.) Disease Control in Crops - Biological and Environmentally Friendly Approaches, pp. 246-256. Wiley-Blackwell, West Sussex, UK.

Birge E.A., 2000. Bacterial and Bacteriophage Genetics, 4th edition. Springer, New York, NY, USA.

Bradley D.E., 1967. Ultrastructure of bacteriophages and bacteriocins. *Bacteriological Reviews* **31**: 230-314.

Carlson K., 2005. Appendix: Working with bacteriophages: Common techniques and methodological approaches. In: Kutter E., Sulakvelidze A. (eds). Bacteriophages: Biology and Applications, pp. 437-494. CRC Press, Boca Raton, Florida, FL, USA.

Civerolo E.L., 1970. Comparative relationships between two *Xanthomonas pruni* bacteriophages and their bacterial host. *Phytopathology* **60**: 1385-1388.

Ellis E.L., Delbrück M., 1939. The growth of bacteriophage. *The Journal of General Physiology* **22**: 365-384.

Erskine J.M., 1973. Characteristics of *Erwinia amylovora* bacteriophage and its possible role in the epidemiology of fire blight. *Canadian Journal of Microbiology* **19**: 837-845.

Flaherty J.E., Harbaugh B.K., Jones J.B., Somodi G.C., Jackson L.E., 2001. H-mutant bacteriophages as a potential biocontrol of bacterial blight of geranium. *HortScience* **36**: 98-100.

Gill J.J., Abedon S.T., 2003. Bacteriophage ecology and plants. APS*net* Features, Online. doi: 10.1094/APSnetFeature-2003-1103.

Gill J.J., Svircev A.M., Smith R., Castle A.J., 2003. Bacteriophages of *Erwinia amylovora*. *Applied and Environmental Microbiology* **69**: 2133-2138.

Hartung J.S., Fulbright D.W., Klos E.J., 1988. Cloning of a bacteriophage polysaccharide depolymerase gene and its expression in *Erwinia amylovora*. *Molecular Plant-Microbe Interactions* **1**: 87-93.

Ignjatov M., Gašić K., Ivanović M., Šević M., Obradović A. Milošević M., 2010. Characterization of *Xanthomonas euvesicatoria* strains pathogens of pepper in Serbia. *Pesticides and Phytomedicine* **25**: 139-149.

Iriarte B.F., Balogh B., Momol M.T., Smith M.L., Wilson M., Jones J.B., 2007. Factors affecting survival of bacteriophage on tomato leaf surfaces. *Applied and Environmental Microbiology* **73**: 1704-1711.

Jackson L.E., 1989. Bacteriophage prevention and control of harmful plant bacteria. U.S. Patent No. 4828999.

Jones J.B., Lacy G.H., Bouzar H., Stall R.E., Schaad N., 2004. Reclassification of Xanthomonads associated with bacterial spot disease of tomato and pepper. *Systematic and Applied Microbiology* **27**: 755-762.

Jones J.B., Jackson L.E., Balogh B., Obradović A., Iriarte F.B., Momol M.T., 2007. Bacteriophages for plant disease control. *Annual Review of Phytopathology* **45**: 245-262.

Kim E.J., Hong J.W., Yun N.R., Lee Y.N., 2011. Characterization of *Bacillus* phage-K2 isolated from chungkookjang, a fermented soybean foodstuff. *Journal of Industrial Microbiology and Biotechnology* **38**: 39-42.

Klement Z., Rudolf K., Sands D.C., 1990. Methods in Phytobacteriology. Akadémiai Kiadó, Budapest, Hungary.

Lang J.M., Gent D.H., Schwartz H.F., 2007. Management of *Xanthomonas* leaf blight of onion with bacteriophages and a plant activator. *Plant Disease* **91**: 871-878.

Liew K.W., Alvarez A.M., 1981. Biological and morphological characterization of *Xanthomonas campestris* bacteriophages. *Phytopathology* **71**: 269-273.

Lin N.T., You B.Y., Huang C.Y., Kuo C.W., Wen F.S., Yang J.S., Tseng Y.H., 1994. Characterization of two novel filamentous phages of *Xanthomonas*. *Journal of General Virology* **75**: 2543-2547.

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 Disease* **67**: 779-781.

Minsavage G.V., Canteros B.I., Stall R.E., 1990. Plasmid-mediated resistance to streptomycin in *Xanthomonas campestris* pv. *vesicatoria*. *Phytopathology* **80**: 719-723.

Obradović A., Mavridis A., Rudolph K., Arsenijevic M., 1999. Characterization of pathogenic bacteria isolated from pepper in Yugoslavia. *Phytomedizin* **29**: 40-41.

Obradović A., Mavridis A., Rudolph K., Arsenijevic M., 2000. Bacterial spot of capsicum and tomato in Yugoslavia. *EPPO Bulletin* **30**: 333-336.

Obradović A., Mavridis A., Rudolph K., Arsenijevic M., Mijatovic M., 2001. Bacterial diseases of pepper in Yugoslavia. In: De Boer S.H. (ed.). Plant Pathogenic Bacteria, pp. 255-258. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Obradović A., Jones J.B., Momol M.T., Balogh B., Olson S.M., 2004a. Management of tomato bacterial spot in the field by foliar applications of bacteriophages and SAR inducers. *Plant Disease* **88**: 736-740.

Obradović A., Mavridis A., Rudolph K., Janse J.D., Arsenijevic M., Jones J.B., Minsavage G.V., Wang J.F., 2004b. Characterization and PCR-based typing of *Xanthomonas campestris* pv. *vesicatoria* from peppers and tomatoes in Serbia. *European Journal of Plant Pathology* **110**: 285-292.

Obradović A., Jones J.B., Momol M.T., Olson S.M., Jackson L.E., Balogh B., Guven K., Iriarte F.B., 2005. Integration of biological control agents and systemic acquired resistance inducers against bacterial spot on tomato. *Plant Disease* **89**: 712-716.

Okabe N., Goto M., 1963. Bacteriophages of plant pathogens. *Annual Review of Phytopathology* **1**: 397-418.

Reanney D.C., Gowland P.C., Slater J.H., 1983. Genetic interactions among microbial communities. In: Slater J.H. (ed.). Microbes in their Natural Environments, pp. 379-422. Cambridge University Press, Cambridge, UK.

Ritchie D.F., Klos E.J., 1977. Isolation of *Erwinia amylovora* bacteriophage from the aerial parts of apple trees. *Phytopathology* **67**: 101-104.

Schnabel E.L., Fernando W.G.D., Meyer M.P., Jones A.L., Jackson L.E., 1999. Bacteriophage of *Erwinia amylovora* and their potential for biocontrol. *Acta Horticulturae* **489**: 649-54.

Schnabel E.L., Jones A.L., 2001. Isolation and characterization of five *Erwinia amylovora* bacteriophages and assessment of phage resistance in strains of *Erwinia amylovora*. *Applied and Environmental Microbiology* **67**: 59-64.

Sutton M.D., Katznelson H., Quadling C., 1958. A bacteriophage that attacks numerous phytopathogenic *Xanthomonas* species. *Canadian Journal of Microbiology* **4**: 493-497.

Svircev A.M., Lehman S.M., Kim W., Barszcz E., Schneider K.E., Castle A.J., 2006. Control of the fire blight pathogen with bacteriophages. In: Zeller W., Ullrich C. (eds). *Proceedings of the 1st International Symposium on Biological Control of Bacterial Plant Diseases, Seeheim/Darmstadt 2005*: 259-261.

Tanaka H., Negishi H., Maeda H., 1990. Control of tobacco bacterial wilt by an avirulent strain of *Pseudomonas solanacearum* M4S and its bacteriophage. *Annals of the Phytopathological Society of Japan* **56**: 243-246.

Thayer P.L., Stall R.E., 1961. A survey of *Xanthomonas vesicatoria* resistance to streptomycin. *Proceedings of the Florida State Horticultural Society* **75**: 163-165.

Tseng Y.H., Lo M.C., Lin K.C., Pan C.C., Chang R.Y., 1990. Characterization of filamentous bacteriophage phi Lf from *Xanthomonas campestris* pv. *campestris*. *Journal of General Virology* **71**: 1881-1884.

Tudor-Nelson S.M., Minsavage G.V., Stall R.E., Jones J.B., 2003. Bacteriocin-like substances from tomato race 3 strains of *Xanthomonas campestris* pv. *vesicatoria*. *Phytopathology* **93**: 1421.

Vidaver A.K., 1976. Prospects for control of phytopathogenic bacteria by bacteriophages and bacteriocins. *Annual Review of Phytopathology* **14**: 451-465.

Watanabe M., Naito K., Kanebo K., Nabasama H., Hosokawa D., 1980. Some properties of *Xanthomonas campestris* pv. *campestris* phage. *Annals of the Phytopathological Society of Japan* **46**: 517-525.

Williams S.T., Mortimer A.M., Manchester L., 1987. Ecology of soil bacteriophages. In: Goyal S.M., Gerba C.P., Bitton G. (eds). Phage Ecology, pp. 157-179. John Wiley & Sons, New York, NY, USA.

Received December 14, 2010
Accepted March 22, 2011