BIOCONTROL POTENTIAL OF BACTERIOPHAGE KΦ1 IN CONTROL OF PEPPER BACTERIAL SPOT

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Introduction

Bacterial spot, caused by *Xanthomonas euvesicatoria* (Figure 1), is widely spread disease of pepper in Serbia. Attempts to control this pathogen with a variety of strategies had limited success. Therefore, alternative approaches were studied in order to develop an efficient and sustainable control strategy for this disease. Use of bacteriophages in plant protection has a great potential and could improve curent control measures.

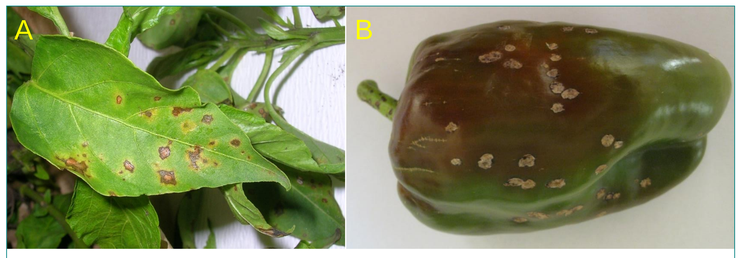

Figure 1. *X. euvesicatoria*. Bacterial spot on pepper leaves (A) and pepper fruit (B). Natural infection.

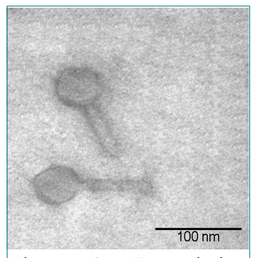

Figure 2. Transmission electron micrographs of phages KΦ1.

Methodology

A bacteriophage strain, designated as KΦ1 (Figure 2), member of the *Myoviridae* family, was isolated from rhizosphere of the diseased pepper plants. An extensive *in vitro* characterization of the phage was followed by studying its efficacy in control of pepper bacterial spot in the greenhouse trials.

Results

The phage showed lytic activity to all *X. euvesicatoria* strains tested and did not lyse other *Xanthomonas* neither less related species (Table 1). The strain KΦ1 is resistant to chloroform, stable in different media and buffers (Figure 3), sustain pH 3 - 9 (Figure 4), and can be stored at 4°C at least two years without decreasing of titer. Copper compounds reduced the phage vitality *in vitro* proportionally to the used bactericide concentration (Figure 5). UV light was detrimental to the phage, but skim milk plus sucrose formulation extended its survival *in vitro* (Figure 6). The phage KΦ1 has a double-stranded 46,077 bp DNA genome with GC content of 62.9% and 66 predicted open reading frames (ORFs). The average gene length was predicted to be 632 nucleotides, and 90.6% of the genome consisted of coding regions. The genome of phage did not encode any transport RNAs and does not carry toxin genes, virulence genes, or genes related to lysogeny, indicating its suitability for a phage therapy.

Table 1. Host range of the bacteriophage KΦ1

Bacterial species	Strain	Origin, host, year of isolation	Source	KΦ1 phage
Xanthomonas euvesicatoria	KBI 116, KBI 117, KBI 118, KBI 119, KBI 120, KBI 121, KBI 123, KBI 124, KBI 125, KBI 126, KBI 127, KBI 128, KBI 129, KBI 130, KBI 131, KBI 132, KBI 133, KBI 134	Serbia, Capsicum annuum, 2015	KBI	+
Xanthomonas euvesicatoria	NCPPB 2968	USA, Capsicum frutescens, 1977	NCPPB	+
Xanthomonas vesicatoria	NCPPB 1423	Hungary, Lycopersicon esculentum, 1957	NCPPB	-
Xanthomonas gardneri	NCPPB 4321	Serbia, Lycopersicon esculentum, 1953	NCPPB	-
Xanthomonas perforans	NCPPB 881	USA, Lycopersicon esculentum, 1991	NCPPB	-
Acidovorax citrulli	NCPPB 3679	USA, Citrullus lanatus, year unknown	NCPPB	-
Erwinia amylovora	KBI 32	Serbia, Cydonia oblonga, 2013	KBI	-
	KBI 68	Serbia, Pyrus communis, 2014	KBI	-
	KFB 687	Serbia, Malus domestica, 2013	KBI	-
	CFBP 1430	France, Pyrus communis, 2010	CFBP	-
Pectobacterium carotovorum ssp. *carotovorum*	KFB 68	Serbia, Brassica oleracea var. capitata, 1999	KFB	-
	KFB 85	Serbia, Apium graveolens, 1998	KFB	-
Dickeya spp.	KBI 05	United Kingdom, Solanum tuberosum, year unknown	KBI	-
Ralstonia solanacearum	NCPPB 4156	The Nederlands, Solanum tuberosum, 1995	NCPPB	
Agrobacterium tumefaciens	C58	USA, Prunus cerasus, 1958	S. Süle	
Clavibacter michiganensis ssp. *michiganensis,*	CFBP 4999	Hungary, Lycopersicon esculentum, 1957	CFBP	-
Clavibacter michiganensis ssp. *sepedonicus*	CFBP 3561	Finland, Solanum tuberosum, 1983	CFBP	-
Pseudomonas syringae pv. *lachrymans*	KFB 214	Serbia, Cucumis sativus, 2007	KFB	-
Pseudomonas syringae pv. *syringae*	GSPB 1142	Germany, Phaseolus sp., 1967	GSPB	-
Pseudomonas fruorescens	B130	Ji et al., 1996	AU	-

+ lysis of bacterial cells (plaque formation), - lack of bacterial cell lysis (no plaque formation)

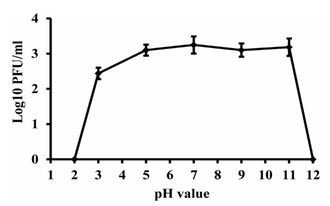

Figure 4. Stability of phage KΦ1 at different pH values during 24 h. Error bars indicate the standard error.

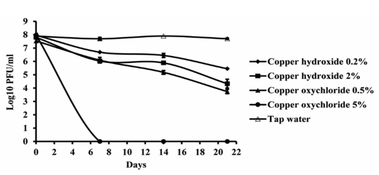

Figure 5. The effect of copper compounds on phage KΦ 1 survival during three weeks. Error bars indicate the standard error.

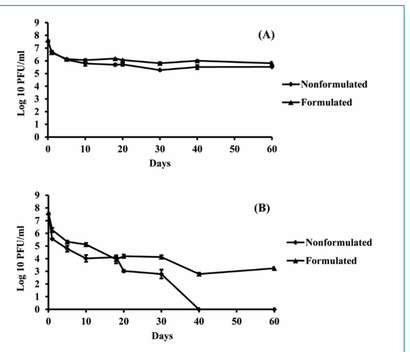

Figure 6. The effect of UV light on survival of KΦ1 phage. Nonformulated and formulated (0.75% skim milk plus 0.5% sucrose) phages were stored during two months either in completely dark conditions (A) or were subjected to a16 h UV light/8 h dark photoperiod (B). Error bars indicate the standard error.

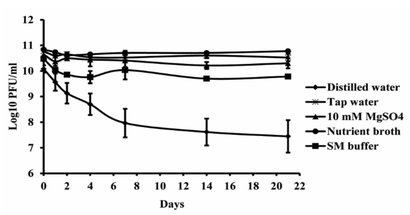

Figure 3. KΦ1 phage survival in different media during three weeks. Error bars indicate the standard error.

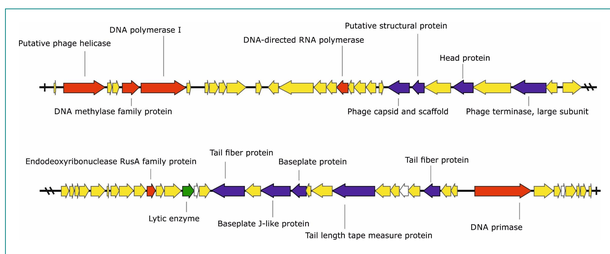

Figure 7. The genome of the bacteriophage KΦ1 (46,077 bp). ORFs coding for proteins involved in DNA metabolism, transcription and translation are marked in red, ORFs coding for proteins involved in phage particle assembly are marked in purple and ORFs coding for enzymes are marked in green. ORFs coding for hypothetical and conserved hypothetical proteins are marked in yellow and ORFs of unknown function are marked in white. Arrows indicate the direction of transcription and translation. The figure was generated using the genome visualization program SnapGene ver. 2.3.4 (http://www.snapgene.com/).

Results of the three repeated greenhouse experiments showed that foliar application of KΦ1 phage (10^8 PFU/ml) significantly reduced the symptom severity of artificially inoculated pepper plants compared to the untreated control (Table 2).

Table 2. The effect of phage KΦ1 treatment in pepper bacterial spot development in greenhouse conditions.

Treatments	Application timing	Average lesion number[y]		
		Experiment 1[x]	Experiment 2	Experiment 3
Phage KΦ1	2 h before inoculation	237 b	302 bc	280 b
Phage KΦ1	2 h before and 15 min after inoculation	157 cb	213 c	182 bc
Phage KΦ1	15 min after inoculation	229 b	358 ab	294 b
Copper-hydroxide* + phage KΦ1	24 h before inoculation; 2 h before inoculation	63 c	41 d	66c
Copper-hydroxide	24 h before inoculation	111 c	106 d	179 bc
Untreated control	None	332 a	422 a	567 a

[x]Concentration of inoculum was 10^8 CFU/ml in experiments 1 and 2, and 10^6 CFU/ml in experiment 3.
[y]Average lesion number per plant 14 days after inoculation. Means followed by different letters within a column are significantly different according to Duncan's multiple range test, P = 0.05 level.
*Kocide 2000, DuPont – active ingredient 53.8% copper-hydroxide. Concentration 0.2%, as recommended by manufacturer was used in all experiments.

Conclusion

Our results showed that phage KΦ1 possesses high specificity and lytic activity to a range of *X. euvesicatoria* strains. The host range, as well as its genomic and other characteristics, indicate that this phage could be an efficient biocontrol agent. Greenhouse trials showed that depending on application frequency, phage treatments can be effectively used in control of pepper bacterial spot. Phage treatment in combination with copper–hydroxide resulted in enhanced disease control on pepper in the greenhouse.

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