Isolation and characterization of the bacteriophages infecting *Xanthomonas arboricola* pv. *juglandis*

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INTRODUCTION

Bacterial blight of walnut caused by *Xanthomonas arboricola* pv. *juglandis* (Xaj) (Figure 1) is one of the most destructive diseases that annually reduces the fruit production in most walnut growing regions worldwide. The disease control is challenging, since abundant use of copper-based compounds in walnut orchards resulted in the emergence of highly copper resistant isolates.

Biological approach in the disease control might be a potential solution and substitute for available bactericides of poor efficacy.

Figure 1. *Xanthomonas arboricola* pv. *juglandis*. Canker on walnut twig (left) and black necrotic spots on walnut fruits (right).

MATERIAL AND METHODS

Nine bacteriophages were isolated from rhizosphere of walnut trees, walnut leaves, fruits or irrigation water in different locations in Serbia (Table 1). The phage isolates were characterized by host range, plaque morphology, thermal inactivation and sensitivity to pH, UV light and chloroform.

Host range was tested by spotting 5 µl of phage suspension onto bacterial lawns of 14 Xaj strains isolated from different localities in Serbia, Italy and Turkey, including three *Xanthomonas arboricola* pv. *pruni* strains (Table 2). Plaque morphology was studied on NYA medium using Xaj strains KFB 75 or KFB 302 as a host. Thermal inactivation point of phage particles was studied by exposing 1 ml of phage suspension to different temperatures (40 – 70°C) in water bath for 10 min. Effect of different pH values on phage viability was studied by 24 h incubation of phage suspension in SM buffer at pH 2, 5, 7, 9 and 11, at room temperature. In order to study effect of UV light irradiation on phage survival, phages were exposed to UV light 254 nm during 2, 5 and 10 min. Sensitivity to chloroform was tested by incubation of phages in suspension containing 20% of chloroform during 1 h.

Table 1. Studied bacteriophage strains – origin, source, and plaque characteristics

Phage	Locality	Source	Plaques
ΦXaj1	Železnik	walnut twigs	clear, ø 1 mm
ΦXaj2	Železnik	walnut leaf	clear, ø 1 mm
ΦXaj3	Barič	soil	clear, ø 1 mm
ΦXaj4	Ostružnica	soil	clear, ø 1 mm
ΦXaj5	Umka	soil	clear, ø 1 mm
ΦXaj6	Šabac	soil	clear, ø 5 mm
ΦXaj7	Šabac	irrigation water	clear, ø 5 mm
ΦXaj8	Šimanovci	soil	clear, ø 5 mm
ΦXaj9	Šimanovci	walnut fruit	clear, ø 1 mm

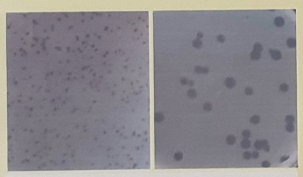

Figure 2. *Xanthomonas arboricola* pv. *juglandis* specific phages. Formation of two types of plaques, ΦXaj5 on Xaj strain KFB 75 as a host (left) and ΦXaj6 on bacterial lawn of the strain KFB302 as a host (right).

Table 2. Host range of the isolated bacteriophages

Bacterial species	Strain	Origin, host, year of isolation	ΦXaj1	ΦXaj2	ΦXaj3	ΦXaj4	ΦXaj5	ΦXaj6	ΦXaj7	ΦXaj8	ΦXaj9
X. a. pv. juglandis	KFB 75	Serbia, *Juglans regia*, 2007	+	+	+	+	+	-	-	-	-
X. a. pv. juglandis	KFB 76	Serbia, *Juglans regia*, 2007	+	+	+	+	+	-	-	-	-
X. a. pv. juglandis	KFB 302	Serbia, *Juglans regia*, 2009	+	+	-	+	+	+	+	+	+
X. a. pv. juglandis	KFB 310	Serbia, *Juglans regia*, 2009	+	+	+	+	+	-	-	-	-
X. a. pv. juglandis	KFB 312	Serbia, *Juglans regia*, 2009	+	+	-	+	+	-	-	-	-
X. a. pv. juglandis	KFB 365	Serbia, *Juglans regia*, 2010	+	+	-	+	+	-	-	-	-
X. a. pv. juglandis	KFB 367	Serbia, *Juglans regia*, 2010	+	+	+	+	+	-	-	-	-
X. a. pv. juglandis	NCPPB 411	New Zealand, *Juglans regia*, 1957	-	-	+	+	-	-	-	-	-
X. a. pv. juglandis	KFB 0251	Italy, *Juglans regia*	-	-	-	+	+	-	-	-	-
X. a. pv. juglandis	KFB 0252	Italy, *Juglans regia*	-	-	-	+	+	+	+	-	-
X. a. pv. juglandis	971	Italy, *Juglans regia*, 2013	-	+	+	+	+	+	-	-	-
X. a. pv. juglandis	1515	Italy, *Juglans regia*, 2018	-	-	-	-	-	-	-	-	-
X. a. pv. juglandis	1560	Italy, *Juglans regia*, 2019	-	+	-	+	+	-	-	-	+
X. a. pv. juglandis	1343	Turkey, *Juglans regia*, 2016	-	+	+	+	+	+	-	-	-
X. a. pv. pruni	NCPPB 272	USA, *Prunus armeniaca*, 1949	-	-	-	-	-	-	-	-	-
X. a. pv. pruni	NCPPB 3744	Brazil, *Prunus persica*, 1990	-	-	-	-	-	-	-	-	-
X. a. pv. pruni	NCPPB 926	South Africa, *Prunus domestica*, 1961	-	-	-	-	-	-	-	-	-

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

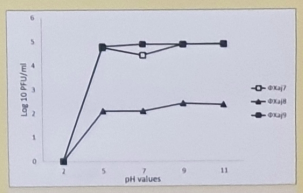

Figure 3. Effect of pH on stability of three phages *in vitro*, after 24 h incubation at room temperature.

RESULTS

A total of nine bacteriophages were isolated during 2019 from different localities in Serbia; 5 phages were isolated from soil, 1 phage from irrigation water and 3 phages originated from plant tissue (Table 1). Phage host range assay showed different lytic activity to tested Xaj strains (Table 2). Five phage isolates lysed at least 7 Xaj strains, while four phage isolates were more specific, infecting 1 to 4 Xaj strains. None of the phages lysed any of the Xap strains tested. Phage ΦXaj4 showed the broadest host range, since this strain was able to infect 13 of 14 tested Xaj strains.

The phage isolates formed clear plaques ca. 1 to 5 mm in diameter, with sharp edges, on lawns of Xaj strains after 24 h incubation (Table 1, Figure 2). Thermal inactivation point of phages was 52 or 54°C. Three tested phages were slightly sensitive to 20% chloroform; after 1 h incubation phage concentration decreased by 0.06-0.8 log units. Tested phages were stable at range of pH 5-11 (Figure 3), but inactivated after 2 min exposure to UV light.

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CONCLUSION

The phage isolates and their characteristics described in this study provide a preliminary results for the further study of phage ecology and application efficacy, in view of the inclusion of phage treatment in walnut bacterial blight management practices.

Lytic life cycle of investigated phage strains indicate their potential in control of walnut bacterial blight. However, sensitivity to UV light could be disadvantage that requires solution, probably by including some UV protectant or development of a formulated phage suspension.

The fact that one of Xaj strain was resistant to all 9 phage strains indicated additional weak point in the phages' host range. Variation of the pathogen strains in susceptibility to the phage strains must be further studied before attempts for controlling the disease in a field were made.