Известия ТСХА, выпуск 6, 2015 год

УДК 632.3.01/.08

BIOLOGICAL PROPERTIES OF BACTERIOPHAGES SPECIFIC TO BLACKROT PATHOGEN OF BRASSICAS *XANTHOMONAS CAMPESTRIS PV. CAMPESTRIS*

VO THI NGOC HA [1], F.S.-U. DZHALILOV [2], A.N. IGNATOV [2, 3]

([1] Russian Timiryazev State Agrarian University; [2] Russian University of People's Friendship; [3] Research Center "PhytoEngineering" Ltd)

Nine isolates of bacteriophages X. campestris pv. campestris were obtained from soil samples collected in the fields under head cabbage crop with outbreak of black rot of brassicas. The isolates had strong lytic effect against 46.2–68.8% of 64 the tested X. campestris pv. campestris strains. Microscopic characterization confirmed that all the bacteriophages were short-tailed phages with the head diameter of 46.6±2.2 nm, with the tail length 134.7±8.3 nm, and were assigned to the family Siphoviridae. Pre-treatment of black-rot pathogen infected seeds with the bacteriophages resulted in significant decrease in the pathogen viable cell concentration in seed extracts, and 2.0–4.1 times reduction of disease rate of cabbage seedlings compared to the control.

Key words: black rot of cabbage, Xanthomonas campestris pv. campestris, the bacteriophages.

Black rot (causing agent *Xanthomonas campestris pv. campestris* (Pammel, 1895; Dowson, 1939) is a devastating disease of brassicas worldwide, and one of main factors reducing cabbage production in the Russian Federation. The disease is present in all regions of Russia where brassicas are cultivated, and can reduce the potential cabbage yield up to 100%, affect product quality, and cause significant loss of head cabbage during winter storage [5, 10].

Biological and copper-based pesticides are the only recommended disease control measures.

However, the emergence of strains resistant to antibiotics-based biologic pesticides and copper ions [6, 8] encourage the search for other control measures, including application of bacteriophages specific for *X. campestris* pv. *campestris*. The first report about bacteriophages applied against plant diseases was dated 1926 [1]. Recent years were characterized by the increasing interest to the use of bacteriophages for plant pathogens control. There are many examples of successful use of bacteriophages to control bacterial black spot (caused by *X. vesicatoria* complex) on tomato plants [2], and bacterial blight (*Erwinia amylovora*) on apple trees [3]. However, we have not found any publications

on practical application of bacteriophages against black rot of brassicas. The goal of our work is to isolate bacteriophages of *X. campestris pv. campestris*, define their biological properties and evaluate their potential efficiency in infected seed treatment.

Material and Methods

The laboratory and greenhouse experiments were conducted at the Laboratory of Plant Protection, Russian Timiryazev State Agrarian University, Moscow. Experiments were carried out in 2014–2015 with collection of strains of *X. campestris* pv. *campestris,* other species of *Xanthomonas*, and the selected isolates of bacteriophages. Soil samples for isolation of bacteriophages were collected in summer 2014 from several cabbage fields where black rot epidemics were previously registered.

Isolation of bacteriophages, validation of specificity, stock concentration and point of thermal inactivation measurement were conducted by conventional methods [1]. Identified bacteriophage isolates were stored in SM buffer (NaCl 5.8 g, $MgSO_4 \times 7H_2O$ 2.0 g, 1M Tris HCl (pH — 7.4) 50 ml and 2% gelatin 5 ml per liter) at 4°C in darkness until future use.

Biological effectiveness of the bacteriophages in treatment of cabbage seeds F_1 Kazatchok infected by black rot was evaluated in greenhouse experiments. Artificial infection of cabbage seeds with *X. campestris* pv. *campestris* strains Tir2 and 276NZ was performed in vacuum as previously described [7]. Then, the seeds were dried at room temperature for 24 hours and treated with phages at a concentration of 10^7 plaque forming units/ml (pfu/ml) with vacuum infiltrartion and then dried again. The control group consisted of infected seeds without bacteriophages treatment. The pathogen was extracted from treated seeds and control seeds on semi-selective medium using previously described method [9]. Counting of yellow mucous starch-clearing colonies characteristic of *X. campestris pv. campestris* was performed two days after the incubation. Part of treated seeds and control seeds was sown in 49-cell cassettes filled with peat substrate and moved to greenhouse with day/night temperature 24/22°C and 16h light day. Black rot development was recorded in 30, 40 and 50 days after sowing.

Statistical analysis of the experimental data was done by MANOVA analysis with comparison of average values by Duncan's test.

Results and Discussion

Eleven bacteriophage isolates were obtained initially from a number of soil samples (Table 1).

Two phage isolates — R2 and R4, lost vitality after 30 day-storage for unknown reasons. The remaining 9 bacteriophage isolates were tested for specificity against a collection of strains of *X. campestris* pv. *campestris* and other species of the genus of *Xanthomonas* in double replication.

According to the obtained data, the isolates of bacteriophages differed in their specificity to the pathogen strains (Table 2). None of the phages affected strains of *X. arboricola* or *X. campestris* pv. *raphani*. Different isolates of bacteriophages were able to infect from 46.2 to 68.8% of strains of *X. campestris* pv. *campestris*.

Moreover, some strains of *X. campestris* pv. *campestris* were resistant to any of the obtained isolates of bacteriophages. This fact indicates that practical application of phages for the black rot control can be done by application of a "cocktail" of phages that covers nearly all pathogen genotypes, including this resistant group. For this purpose the selection of bacteriophages that are lytic to the found resistant group of bacterial strains is required.

Table 1

Bacteriophages isolates of *X. campestris pv. campestris* (Xcc)

Bacterio-phages	Host Strain of Xcc	Source	Origin of Sample
R2	Ram 1-1	Soil	Moscow, Moscow State Agrarian University — MSKHA, Plant Protection Lab.
R4	Ram 1-1	Soil	Moscow, Moscow State Agrarian University — MSKHA, Plant Protection Lab.
Tir2'	Tir 2	Soil	Moscow region, Lukhovitsy distr., "Soin" farm
Tir2X1	Tir 2	Soil	Moscow region, Lukhovitsy distr., "Soin" farm
Tir2X2	Tir 2	Soil	Moscow region, Lukhovitsy distr., "Soin" farm
Tir2DB1	Tir 2	Soil	Moscow region, Lukhovitsy distr., "Soin" farm
DB1'	DB1	Soil	Moscow region, Lukhovitsy distr., "Soin" farm
Tr1'	Tr1	Soil and piles	Research Institute of Agriculture, Tiraspol, Moldova
B1'	B1	Soil and piles	Research Institute of Agriculture, Tiraspol, Moldova
R3-1	Ram 3-1	Soil and piles	Research Institute of Agriculture, Tiraspol, Moldova
T2	Tir2	Soil and piles	Research Institute of Agriculture, Tiraspol, Moldova

Table 2

Specificity of bacteriophage isolates tested on the collection *X. campestris* pv. *campestris* strains (*Xcc*)

Xcc strain	Bacteriophage isolates								
	Tir 2'	Tir 2-X1	Tir 2-X2	Tir 2-DB1	DB1	T2	B1'	Tr1'	R3-1
DK-1	+	+	+	+	+	+	+	+	+
DK-2	+	+	+	+	+	+	+	+	+
DK-3	+	+	+	+	+	+	+	+	+
DV-1	–	–	–	–	–	–	–	–	–
DV-2	–	–	–	–	–	–	–	–	–
DV-3	–	–	–	–	–	–	–	–	–
Ram — 1-1	+	+	+	+	+	+	+	+	+
Ram — 1-2	+	+	+	+	+	+	+	+	+
Ram — 1-3	+	+	+	+	+	+	+	+	+
Ram — 2-1	+	+	+	+	+	+	+	+	+

Продолжение табл. 2

Xcc strain	Bacteriophage isolates								
	Tir 2'	Tir 2-X1	Tir 2-X2	Tir 2-DB1	DB1	T2	B1'	Tr1'	R3-1
Ram — 2-2	+	+	+	+	+	+	+	+	+
Ram — 2-3	+	+	+	+	+	+	+	+	+
Ram — 3-1	+	+	+	+	+	+	+	+	+
Ram — 3-2	+	+	+	+	+	+	+	+	+
Ram — 3-3	+	+	+	+	+	+	+	+	+
Ram — 4-1	+	+	+	+	+	+	+	+	+
Ram — 4-2	+	+	+	+	+	+	+	+	+
Ram — 4-3	+	+	+	+	+	+	+	+	+
B-1	+	+	+	+	+	+	+	+	+
B-2	+	+	+	+	+	+	+	+	+
B-3	+	+	+	+	+	+	+	+	+
Tir1	+	+	+	+	+	+	+	+	+
Tir2	+	+	+	+	+	+	+	+	+
Tir3	+	+	+	+	+	+	+	+	+
XY-1-1	–	–	–	–	–	–	–	–	–
XY 1-2	–	–	–	–	–	–	–	–	–
XY 2-1	–	–	–	–	–	–	–	–	–
XY 2-2	–	–	–	–	–	–	–	–	–
177NZ	–	–	–	–	+	+	+	+	+
276 NZ	+	+	+	+	+	+	+	+	+
306 NZ	–	–	–	–	+	+	+	+	+
Eruca	–	–	–	–	–	–	–	–	–
Xok-1	+	+	+	+	+	–	–	–	–
Tr1	–	–	–	–	–	+	+	+	+
Tr2	–	–	–	–	–	+	+	+	+
Tr3	–	–	–	–	–	–	–	+	+
Tr4	–	–	–	–	–	–	–	+	+

Продолжение табл. 2

Xcc strain	Bacteriophage isolates								
	Tir 2'	Tir 2-X1	Tir 2-X2	Tir 2-DB1	DB1	T2	B1'	Tr1'	R3-1
Tr5	–	–	–	–	–	–	–	–	–
Tr6	–	–	–	–	–	–	–	–	–
$Л_1$	–	–	–	–	–	–	–	–	–
A5	–	–	–	–	–	–	–	+	+
ex 528	–	–	–	–	–	+	+	+	+
Tlo-1	+	+	+	+	+	+	+	+	+
Tlo-2	+	–	+	–	–	+	+	+	+
Tlo-3	–	–	+	–	–	+	+	+	+
Tlo-4	+	+	+	+	+	+	+	+	+
Tlo-5	+	–	+	–	–	+	+	+	+
AF-2	–	–	–	–	–	–	–	+	+
11390	–	–	–	–	–	+	+	+	+
11392	–	–	–	–	–	+	+	+	+
042981	–	–	–	–	–	–	+	+	+
33437	+	+	+	+	+	+	+	+	+
11386	–	–	–	–	–	–	–	–	–
Bul-K	–	–	–	–	–	–	–	–	–
Xn-13	+	+	+	+	+	–	–	–	+
Bun-2	–	–	–	–	–	–	–	+	+
Bel-2	–	–	–	–	–	–	–	–	–
Bel-3	–	–	–	–	–	–	–	–	–
Bel-8	–	–	–	–	–	–	–	–	–
Bel-9	–	–	–	–	–	–	–	–	–
Dasch-2	+	+	+	+	+	+	+	+	+
Xn18a	+/–	+/–	+/–	+/–	+/–	+	+	+	+
SM 17	+/–	+/–	+/–	+/–	+/–	–	–	–	–

Окончание табл. 2

Xcc strain	Bacteriophage isolates								
	Tir 2'	Tir 2-X1	Tir 2-X2	Tir 2-DB1	DB1	T2	B1'	Tr1'	R3-1
Th 266	–	–	–	–	–	–	–	–	–
Susceptible phage reaction, %	50.0	46.2	51.6	46.2	50.0	57.8	59.4	67.2	68.8
11346	–	–	–	–	–	–	–	–	–
11348	–	–	–	–	–	–	–	–	–
10836	–	–	–	–	–	–	–	–	–
1392	–	–	–	–	–	–	–	–	–
3004	–	–	–	–	–	–	–	–	–
5001	–	–	–	–	–	–	–	–	–
Susceptible phage reaction, %	0	0	0	0	0	0	0	0	0

Note: strains 11346, 11348, 10836 — *X. arboricola*, USA; strains 1392, 3004 — *X. arboricola*, Russia, strain 5001 — *X. campestris* pv. *raphani*, Russia.
"+" — susceptible reaction, "–" — resistant, "+/–" — partly susceptible.

Before the isolates of bacteriophages were applied in experiments on plant protection, we evaluated the efficient concentration of the virus. To this end it was necessary to determine the original concentration of the stock suspensions of bacteriophages.

The obtained results show that the stock concentrations of different phages varied from 1.2×10^{9} to 1.3×10^{11} pfu/ml. The temperature of inactivation for the isolates Tir2X1 and Tir2X2 was 74°C, Tir2' and DB1' — 75°C; and Tir2DB1 — 76°C (Table 3).

Table 3

Concentrations of stock suspension of bacteriophages and their temperature of thermal inactivation

Bacteriophage	Isolate Concentration in Stock, pfu/ml	Termal Inactivation, °C
Tir2'	$5*10^{10}$	75
Tir2X1	$2*10^{11}$	74
Tir2X2	$8*10^{10}$	74
Tir2DB1	$1.3*10^{11}$	76
DB1'	$1.2*10^{9}$	75

Electronic microscope study of the bacteriophages morphology showed that they were tailed phages with a head diameter of 46.6±2.2 and the length of the tail 134.7±8.3 nm. Based on these data, the phages belong to the *Siphoviridae* family of long tailed phages [1].

Treatment of infected seeds of cabbage with bacteriophages resulted in a significant decrease in the content of viable cells of the pathogen in the seed extracts. In cases of bacteriophage seed treatment, infection of seedlings was significantly lower compared to control plants during all three scoring times. On the opposite, the use of the seeds infected with Tir2 strain resulted in significant disease progress on seedlings in 50 days after sowing up to 86.9% of infected plants, while seed treatment with bacteriophage DB1' slowed down the infection rate to 21.4%. The biological efficiency of treatment with bacteriophage was about 75.4%. Similar data were obtained for seeds inoculation with strain 276NZ (Table 4).

Table 4

Concentration of *X. campestris* pv. *campestris* in seed extracts and black rot disease rate on cabbage seedlings F_1 Kazatchok after treatment of seeds by bacteriophages

Xcc Strain	Phage Isolate	Xcc Number in Seed Extract (*10^4 CFU/ml)	Disease Rate on Seedlings, % Days after Sowing		
			30	40	50
Tir 2	Tir2'	34.0b	30.2c	30.2de	36.0bc
	Tir2X1	42.5b	26.7bc	31.1e	44.4cd
	Tir2X2	45.0b	16.6abc	23.3bcde	33.3abc
	Tir2DB1	55.5b	12.9ab	26.1cde	33.1abc
	DB1'	32.5ab	10.8ab	15.5abcd	21.4ab
	control	650.0d	67.9e	81.1g	86.9e
276NZ	Tir2'	40.5b	6.50a	9.2ab	14.1a
	Tir2X1	8.0a	9.8ab	9.8ab	23.0ab
	Tir2X2	42.5b	9.8ab	9.8ab	14.6a
	Tir2DB1	43.5b	7.7a	12.2abc	14.4a
	DB1'	48.b	7.9a	7.9a	16.1a
	control	106.5c	51.8d	51.8h	57.9d

X. campestris pv. *campestris* has a wide range of host brassica plants and occur in a range of climatic and soil conditions. It shows great phenotypic and genotypic diversity between strains. Therefore, determining the host range of each bacteriophage isolate should be done before deciding on the member isolates to be used in the phage cocktail. Determining the host range of each phage allows to design a phage cocktail capable of

lysing all known pathogenic strains involved in the disease. For instance, Bouzar et al. [4] used 26 bacteriophages to type approximately 100 Xanthomonas euvesicatoria strains isolated from various countries in the Caribbean region including Central America, and identified at least 26 different phage lysis patterns.

Thus, the 9 isolates of bacteriophages obtained from soil samples were specific to multiple strains of black rot pathogen. The use of phages for treatment of contaminated infected seeds resulted in significant decrease in the concentration of viable cells of the pathogen and in reduced disease rate (2–4 times) comparing to the untreated control. It will be possible to create an efficient cocktail of phages for plant protection after finding additional isolates specific to the group of pathogen strains resistant to the already studied 9 isolates of phages. In this case, the use of bacteriophages together with the biological pesticides based on raw antibiotic mixtures may prevent the rapid accumulation of resistance to biopesticides among isolates of the pathogen.

The authors acknowledge Dr. P. A. Ivanov and Dr. I.V. Petrunya (Lomonosov Moscow State University) for technical assistance in electronic microscopic study of bacteriophages and Dr. G. Monakhos (Breeding station by N.N. Timofeev, Moscow) for assistance in collecting soil samples.

References

1. Bacteriophages: biology and practical applications / ed. by E. Cutter, A. Sulakvelidze // translation from English, scientific. edited by A. Letarov. Moscow: Scientific world. 2012. 640 p.

2. *Balogh B., Jones J.B., Momol M.T., Olson S.M., Obradovic A., King P., Jackson L.E.* Improved efficacy of newly formulated bacteriophages for management of bacterial spot on tomato // PlantDisease. 2003. V. 87. P. 949–954.

3. *Boule J., Sholberg P.L., Lehman S.M., O'Gorman D.T., Svircev A.M.* Isolation and characterization of eight bacteriophages infecting *Erwinia amylovora* and their potential as biological control agents in British Columbia, Canada // Can. J. Plant Pathol. 2011. V. 33. P. 308–317.

4. *Bouzar H., Jones J.B., Stall R.E., Louws F.J., Schneider M., Rademaker J.L.W., de Bruijn F.J. and Jackson L.E.* 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. 1999. V. 89. № 4. P. 328–335.

5. *Dzhalilov F.S., Vo Thi Ngoc Ha.* Protection of cabbage from diseases during the growing season // Potatoes and vegetables. 2014. № 1. P. 20–23.

6. *Marco G.M., Stall R.E.* Control of bacterial spot of pepper initiated by strains of *Xanthomonas campestris pv. vesicatoria* that differ in sensitivity to copper // Plant Disease. 1983. V. 67. P. 779–781.

7. *Mazurin E., Dzhalilov F.S., Ignatov A.N., Varitses Y.A.* Improvement of diagnosis of infection of seeds of cabbage, the causative agent of black rot by enzyme immunoassay // Izvestia of Timiryazev Agricultural Academy. 2009. Vol. 1. P. 66–72.

8. *McGhee G.C., Guasco J., Bellomo L.M., Blumer-Schuette S.E., Shane W.W., Brown A., Sundin G.W.* Genetic analysis of streptomycin-resistant (SmR) strains of *Erwinia amylovora* suggests that dissemination of two genotypes is responsible for the current distribution of SmR *E. amylovora* in Michigan // Phytopathology. 2011. V. 101. P. 182–191

9. *Vo Thi Ngoc Ha, Dzhalilov F.S.* Antibacterial activity of essential oils and their use for disinfection of cabbage seeds from black rot // Izvestia of Timiryazev Agricultural Academy. 2014. Vol. 6. P. 59–68.

10. *Vo Thi Ngoc Ha, Dzhalilov F.S., Ignatov A.N.* Sustainability assessment various hybrids of white cabbage to black rot // Bulletin PFUR. Series agronomy and animal husbandry. 2015. № 2. P. 7–15.

БИОЛОГИЧЕСКИЕ СВОЙСТВА БАКТЕРИОФАГОВ ВОЗБУДИТЕЛЯ СОСУДИСТОГО БАКТЕРИОЗА КАПУСТНЫХ *XANTHOMONAS CAMPESTRIS PV. CAMPESTRIS*

ВО ТХИ НГОК ХА[1], Ф.С. ДЖАЛИЛОВ[1], А.Н. ИГНАТОВ[2, 3]

([1] РГАУ-МСХА имени К.А. Тимирязева;
[2] Российский университет дружбы народов;
[3] Исследовательский Центр «ФитоИнженерия»)

Из образцов почвы, полученных с полей, где наблюдалось сильное развитие сосудистого бактериоза капусты, выделено 9 изолятов бактериофагов X. campestris pv. campestris. Изоляты бактериофагов оказывали литическое действие на 46,2–68,8% от 64 испытанных штаммов фитопатогена. Они имели форму хвостатых фагов с диаметром головки 46,6±2,2, длиной хвоста 134,7±8,3 нм и были отнесены к семейству Siphoviridae. Использование бактериофагов для предпосевной обработки зараженных семян привело к значительному снижению концентрации жизнеспособных клеток патогена в экстракте семян и к уменьшению зараженности рассады сосудистым бактериозом в 2,0–4,1 раза по сравнению с контролем.

Ключевые слова: сосудистый бактериоз капусты, Xanthomonas campestris pv. campestris, бактериофаги.

Vo Thi Ngok Ha — PhD Student of the Department of Plant Protection, Russian Timiryazev State Agrarian University (127550, Moscow, Timiryazevskaya street, 49; tel.: +7 (499) 976-02-20; e-mail: ngochavo.88@gmail.com).

Dzhalilov Fevzi Seid-Umerovitch — Doctor of Biological Sciences, professor, Head of Plant Protection Laboratory, Russian Timiryazev State Agrarian University (127550, Moscow, Timiryazevskaya street, 49; tel.: +7 (499) 976-12-79; e-mail: labzara@mail.ru).

Ignatov Alexander Nikolaevich — Doctor of Biological Sciences, professor of Agro-biotechnology Department, Agro-technological Institute of Russian People's Friendship University (117198, Moscow, ul. Miklukho-Maklaya, 8; tel.: (916) 671-21-47; an.ignatov@gmail.com).

Во Тхи Нгок Ха — асп. кафедры защиты растений РГАУ-МСХА имени К.А. Тимирязева (127550, Москва, ул. Тимирязевская, 49; тел.: (499) 976-02-20; e-mail: ngochavo.88@gmail.com).

Джалилов Февзи Сеид-Умерович — д. б. н., проф., зав. лабораторией защиты растений РГАУ-МСХА имени К.А. Тимирязева (127550, Москва, ул. Тимирязевская, 49; тел.: (499) 976-12-79; e-mail: labzara@mail.ru).

Игнатов Александр Николаевич — д. б. н., проф. кафедры агробиотехнологии аграрного факультета Российского университета дружбы народов (117198, Москва, ул. Миклухо-Маклая, 8; тел.: (916) 671-21-47; e-mail: an.ignatov@gmail.com).