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Technological aspects of using bacteriophage Ayka to protect soybeans against bacterial wilt

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Abstract. The article presents a study of the use of a protector for rhizobial bacteria as a possible preparative form for the Ayka bacteriophage to effectively combat bacterial wilt of soybean, as well as the possibility of phage transport from the soil to soybean plants through the root system. The study showed that the use of the Premax protector, which is part of the Atuva, WS inoculant, ensures a longer preservation of the bacteriophage titer compared to water during storage of the working solution: the bacteriophage titer in the working solution on the 20th day was 7.2% of the initial titer in the variant with the protector and 0.6% in the variant with an aqueous solution. And also during seed treatment: 24.5% of the initial titer in seed washes in the variant with the protector and 0.6% in the variant with an aqueous solution on the 15th day after the start of the experiment. In addition, the effect of phage translocation into soybean plants from the soil was found in case of root damage, as well as in variant with rapid soil moisture fluctuations.

1 Introduction

Among the factors affecting the yield and quality of soybean in Russia and the world, bacterial diseases pose a serious danger. One of the common bacterial diseases of this crop in our country is bacterial wilt or bacterial tan spot of soybean [1, 2].

The causative agent of the disease is the gram-positive aerobic bacteria *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (Hedges) Collins & Jones. The pathogen affects the vascular system and causes symptoms such as wilting, growth retardation, and death of seedlings. Chlorotic areas with a yellow halo form on the leaves, the tissue of which eventually becomes necrotic. The disease spreads with seed material and precipitation. The pathogen can enter through wounds, natural openings, and through damage to the roots caused by nematodes [3]. The pathogen persists in plant debris and reservoir weeds. In addition to soybeans, the host plants of the pathogen include common beans, mung beans, cowpeas, and other legumes [4].

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One of the problems in combating against bacterial diseases of plants is the development of resistance to antibiotics and chemicals in the absence of alternation of pesticides and violation of the principles of integrated plant protection [5].

In this regard, one of the alternatives to chemical agents could be bacteriophages – viruses that kill bacteria, have evolved together with their «host» and are safe for the environment [6, 7].

Bacteriophages have already found their use in medicine, and in the last decade, with the development of organic farming and the expansion of the use of biological plant protection products, the issue of their use in plant protection has been actively studied. Bacteriophages are highly specific, safe for humans, animals and other non-target objects. The conducted studies on the use of bacteriophages for protection against vascular bacteriosis of cabbage, blackleg of potatoes and other bacteriosis have shown the prospects of their use [7].

Previous studies have shown that the Ayka bacteriophage, which belongs to the φ29-like (*Salasmaviridae*) viruses, is capable of significantly reducing the development of bacterial wilt of soybean both when treating leaves and when treating seeds [9]. However, the effectiveness of bacteriophages can be significantly reduced due to the impact of environmental factors: ultraviolet light, temperature, pH, and combined treatment with other drugs. Accordingly, measures are needed to protect phages from these effects [6, 9]. Some solutions to this problem may include the use of bioprotectors, combining the process of seed inoculation and phage treatment, and soil treatment with a phage solution [6].

The objectives of the study included determining the effect of the Premax protector on the preservation of the Ayka bacteriophage, the possibility of combining the process of inoculation of soybean seeds with bacteriophage treatment, and determining the conditions for the translocation of the Ayka bacteriophage into the soybean plants from the soil.

2 Materials and methods

2.1 Bacteria, bacteriophages and cultivation conditions

The *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* CFBP 3418 strain (French Collection of Phytopathogenic Bacteria CFBP; Beaucouzé, France) was used for the experiments; the bacteria were stored in a 15% glycerol solution at -72°C. Bacteria were cultivated at +28°C on Petri dishes with YD agar medium (YDC without calcium carbonate). The phage titer was calculated using the two-layer agar method, where the upper layer contained 0.7% agar.

Bacteriophage Ayka (universal nomenclature name - vB_CffP_Ayka) was previously isolated from soil in the Penza region and characterized by us in [8]. The stock phage concentrate was stored in SM buffer at +4°C.

2.2 Obtaining lysate, isolation and concentration of bacteriophages

The lysate was prepared and concentrated according to [10-11] in liquid YD medium. For this purpose, 5 ml of the starting culture with $OD_{600\ nm}$~0.15 prepared the day before were added to 400 ml of the medium and cultuvated on a shaker at +28°C and 180 rpm for three hours. Then 100 μl of the viral particle suspension were added and left overnight. After 12 hours, the lysate was cleared of bacterial cells by centrifugation for 10 minutes at 8000 rpm in 50 ml Eppendorf tubes. NaCl and PEG were added to the resulting supernatant to precipitate viral particles and left overnight at +4°C. The supernatant was then centrifuged for 20 minutes at 8000 rpm, and the precipitate was dissolved in SM buffer and purified using chloroform. To concentrate, the solution obtained after purification was centrifuged in 1.5 ml

tubes for 3 hours at 20,000 x g, the supernatant was poured off, and the precipitate was dissolved in SM buffer so that the total sample volume did not exceed 1 ml [12].

2.3 Analysis of the preservation of phage particles in the protector and water

In this experiment, a protector for rhizobial bacteria was tested as a possible preparative form for the phage. The protector Premax from the inoculant Atuva, WS (manufacturer Syngenta Russia) was used in the experiment. 9 ml of the protector were collected in a 50 ml test tube and mixed with 1 ml of the phage suspension with a concentration of $1*10^{10}$ PFU/ml, so that the concentration in the solution was $\sim 1*10^{9}$ PFU/ml, and mixed for 3 minutes on a microcentrifuge shaker. The same was done in the version with sterile distilled water instead of the protector. The experiment was repeated three times.

To create conditions similar to those in a warehouse in the spring before the seeds are treated with the inoculant, the test tubes were placed in a thermostat at +13°C. Samples of 1 ml were taken immediately after mixing the solution (0 h), after 2, 12, 24 hours and on the 7th, 15th and 20th day. The collected sample was cleared of bacteria by adding 100 µl of chloroform and centrifuged at 13,000 rpm. The resulting supernatant was transferred to 1.5 ml test tubes. After 10-fold serial dilutions of the samples, 10 µl were applied to the upper agar using a dispenser.

2.4 Analysis of phage preservation on seeds

In this experiment, we analyzed the phage survival rate when treating soybean seeds with it in a mixture with a protector. The working solution of the inoculant was prepared based on the manufacturer's recommendations: Atuva 2 l/t + Premax 0.5 l/t, the working solution dosage rate is 8 l/t. Part of the water that should be added into the working solution of the inoculant was replaced with a phage suspension in SM buffer, based on a ratio of 1:9, so that the virus concentration was $13*10^{7}$ PFU/ml. For treating seeds with phage solution in sterile water the working solution was prepared similarly: 1 part of the phage solution in SM buffer to 9 parts of water; the virus concentration was $18*10^{7}$ PFU/ml. To assess the phage survival on soybean seeds, Belgorodskaya-7 cultivar seeds were used. 10 grams of seeds were placed in a 50 ml test tube, 80 µl of the working solution were added, mixed in a microcentrifuge-vortexer and placed in a thermostat at +13°C with open lids to dry the seeds. To obtain seed washings, 10 ml of SM buffer were added to the seeds, mixed for 2 minutes in a microcentrifuge-vortexer and left for 30 minutes in an upright position at room temperature [13, 14]. Samples of 2 ml were taken immediately after seed treatment (0 h), after 2, 12, 24 hours, 7 and 15 days. 1 ml of sample was transferred to 1.5 ml test tubes, treated with chloroform, serial dilutions were carried out and applied to the medium with top agar, as indicated earlier. The experiment was repeated three times.

2.5 Analysis of phage preservation on seeds during germination

The seeds were treated with an inoculant containing the phage at a concentration of $\sim 1*10^{7}$ as described above. The control variant was treated with an equivalent volume of sterile water, the experiment was repeated three times. The seeds were germinated using the paper roll method for 12 days, until the formation of the first true leaves. To determine whether the bacteriophage enters the seeds during their germination, 10 sprouts were selected from each repetition and surface sterilized with a 70% bleach solution (17% sodium hypochlorite) for 15-17 minutes, then thoroughly washed under running tap water and left drying on paper towels until completely dry. The control plants, which the seeds were treated with water, were sprayed with 5 ml of bacteriophage solution ($\sim 1*10^{6}$ PFU/ml) from a hand sprayer (10

plants from each replicate), and then surface sterilized as described earlier. The negative control included treatment of plants with phage suspension without subsequent surface sterilization. Then the seedlings were placed in stomacher bags, 80-100 ml of SM buffer were added and placed in a stomacher paddle blender for 3-5 minutes. Then 1 ml of samples were taken, treated with chloroform and plated on the upper agar.

2.6 Phage translocation into plants during fluctuations in soil moisture and maintenance of moisture at a certain level

Soybean plants were grown in a turf-perlite mixture at +24°C on shelves with constant lighting. In the R1 growth phase, soybeans were transplanted into plastic pots containing 500 g of soil, their total mass was weighed and watered to the required irrigation mass. The experiment included the following variants:
1) Control: Maintaining humidity at 50-55% of the total moisture capacity (TMC) without damaging the roots;
2) Maintaining humidity at 50-55% of TMC + damaging the roots;
3) Humidity fluctuations: 50-55% of TMC to wilting point humidity (WH) to 50-55% of TMC, without damaging the roots;
4) Humidity fluctuations: 50-55% of TMC to WH humidity to 80% of TMC, without damaging the roots

Watering of plants with phage solution ($1*10^{11}$ PFU/ml) in volume of 50 ml was carried out 12 days after transplantation from turf-perlite mixture to soil. In the variant with damage to roots, plants were carefully removed from the soil, their roots were washed in water and kneaded with tweezers until a characteristic crunch was heard, thereby causing damage. Plants together with pots were placed in tightly closed zip-bags to increase the intensity of transpiration. Each variant included 4 plants in triplicate. Leaves, starting from the lower ones, were collected on the 1st, 2nd, 4th and 6th day after watering with bacteriophage. They were then homogenized with a sterile plastic pestle in 2 ml test tubes, 1 ml of SM buffer and 100 μl of chloroform were added, the plant tissues were precipitated in a centrifuge at 12,000 rpm, the supernatant was collected and the presence of phage particles was determined as described previously [15, 16].

2.7 Determination of soil moisture

Before conducting the experiment with soil moisture fluctuations, its initial moisture content was determined using the classic thermostat-weight method. For this, 100 g of soil was collected in an aluminum weighing box and placed with the lid open in a thermostat for 6 hours at 105°C. After weighing the weighing box, it was sent for control drying for 2 hours, followed by measuring its mass. The mass of absolutely dry soil was determined, and the initial soil moisture content was calculated, which was 5.6%. Then 500 g of soil were collected in pots and the irrigation mass of them was determined using a known method [17]. The total moisture capacity of the soil, according to our definition, was equal to 50% of the absolutely dry soil, the initial soil moisture content was 5.6%, and irrigation was planned to be carried out up to 50% of the total moisture capacity. This means:
The soil moisture level in the pots should be

$$(50*50):100 = 25\% \qquad (1)$$

of the absolutely dry soil. The mass of absolutely dry soil in the pot is

$$(500*100):105{,}6 = 473 \text{ gm} \qquad (2)$$

The mass of water when moistened to 25% of absolutely dry soil (50% of total moisture capacity) equals

$$(473*25):100 = 118 \text{ gm} \quad (3)$$

By summing this value with the total mass of the pot and the plant, we obtained the irrigation mass when maintaining the humidity at a level of 50-55% of the full moisture capacity. The irrigation mass for 80% of the full moisture capacity was determined in the same way. The humidity of stable wilting, at which signs of plant wilting appear, was determined experimentally, using the weight method described above, measuring the mass of pots with plants that showed signs of wilting. It ranged from 20% of the full moisture capacity and below. The soil dried out naturally until the humidity of stable wilting, and the entire cycle of humidity fluctuations took 10-12 days.

3 Results and discussion

3.1 Changes in phage titer in Premax protector and water during storage of solutions

To assess the preservation of the bacteriophage, the variant with the addition of the phage to the Premax protector was compared with the control variant with sterile distilled water. 1 ml of the phage suspension at a concentration of $\sim 1*10^9$ PFU/ml was added to 9 ml of the Premax protector and 9 ml of sterile distilled water. In both variants, there was a gradual decrease in the titer, starting from the 2nd hour, but in the variant using the protector, the decrease was slower, reaching a value of $8.9*10^7$ by the 20th day, which was 7.2% of the initial titer. In the variant with water, the concentration of viral particles dropped significantly in the period from 15 to 20 days, and also varied significantly across variants, reaching 0.6% of the initial (Table 1).

Table 1. Dynamics of changes in the titer of phage Ayka.

	Water			Premax		
Expo-sition	Average titer value, PFU/ml	Standart deviation	Titer in % of initial	Average titer value, PFU/ml	Standart deviation	Titer in % of initial
0 h	$109.0*10^7$	22.9	100.0	$122.7*10^7$	27.1	100.0
2 h	$91.3*10^7$	26.2	83.8	$110.3*10^7$	27.6	89.9
12 h	$28.9*10^7$	3.0	26.5	$29.7*10^7$	1.5	24.3
24 h	$15.7*10^7$	3.9	14.4	$26.1*10^7$	2.0	21.3
7 days	$2.8*10^7$	1.3	2.6	$16.8*10^7$	3.6	13.7
15 days	$2.2*10^7$	2.1	2.0	$10.5*10^7$	3.2	8.5
20 days	$62.6*10^5$	65.7	0.6	$8.9*10^7$	2.8	7.2

3.2 Phage titer during seed treatment

Addition of Premax ensured greater preservation of the phage on the surface of the seeds, compared to the aqueous solution. Thus, analysis of seed washes showed that the titer of the bacteriophage on the 15th day after treatment with Atuva was 24.5% ($30.3*10^4$ PFU/ml) of the initial, while in the variant using an aqueous solution of the phage - only 0.9% ($5.2*10^3$ PFU/ml) (Table 2).

3.3 Assessment of the probability of bacteriophage translocation into seeds during germination

Germination of seeds treated with the bacteriophage mixed with the Atuva inoculant using the paper roll method with subsequent surface sterilization of the sprouts, their grinding and homogenization in a mixture with SM buffer did not show the presence of viable phage particles. Based on this, it can be concluded that the bacteriophage does not translocate from the surface of the seeds into the sprouts during their germination.

Table 2. Comparison of phage titer in washes from treated seeds.

	Atuva			Water		
Expo-sition	Average titer value, PFU/ml	Standart deviation	Titer in % of initial	Average titer value, PFU/ml	Standart deviation	Titer in % of initial
0 h	$30.3*10^4$	1.5	100.0	$60*10^4$	17.3	100.0
2 h	$24.3*10^4$	6.6	80.2	$16.7*10^4$	8.0	27.8
12 h	$7.9*10^4$	2.8	26.0	$10.8*10^4$	10.3	18.1
24 h	$17.3*10^4$	7.6	57.1	$2.7*10^4$	1.0	4.6
7 days	$7.3*10^4$	2.3	24.17	$2.3*10^3$	2.5	0.4
15 days	$7.4*10^4$	3.5	24.5	$5.2*10^3$	5.92	0.9

3.4 Detection of bacteriophage translocation into plants with artificial damage to roots, with changes in soil moisture

In the leaves of plants which roots were not artificially damaged and constant soil moisture was maintained at 50% of the total moisture capacity, the absence of phage particles was noted in all samples. Phage translocation was detected in both variants with humidity fluctuations, but its highest concentration in the leaves was achieved in the variant with artificial damage to the roots using tweezers (Fig. 1, Table 3).

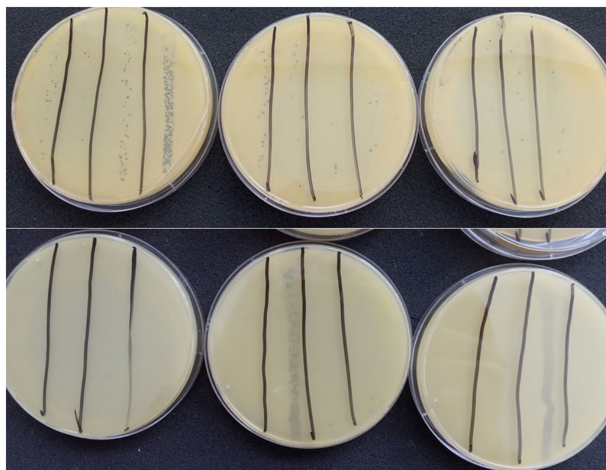

Fig. 1. Comparison of phage titer with maintained constant soil moisture and root damage. Upper row - 1st day after watering with phage solution. Lower row - 6th day after watering with phage solution

Table 3. Phage titer in soybean leaves during translocation from soil to plants, PFU/ml ± standard deviation

Incubation period, days	Maintaining humidity at 50-55% of TMC without damaging the roots	Maintaining humidity at 50-55% of TMC without damaging the roots	Humidity fluctuations 50% of TMC- WH-50% of TMC, without damaging the roots	Humidity fluctuations 50% of TMC- WH-80% of TMC, without damaging the roots
1	0 ± 0	$101.8*10^2 \pm 156.1$	$4.5*10^2 \pm 4.7$	$2.5*10^2 \pm 2.2$
2	0 ± 0	$87.4 *10^2 \pm 142.1$	$1.0*10^2 \pm 0.76$	$14.8*10^2 \pm 18.6$
4	0 ± 0	$16.3*10^2 \pm 7.4$	$0.5*10^2 \pm 0.5$	$1.1 *10^2 \pm 1.4$
6	0 ± 0	$170.4 *10^2 \pm 146.2$	$31.5 *10^2 \pm 48.9$	0 ± 0

4 Conclusion

The conducted studies have shown ways to improve the efficiency of bacteriophage therapy technology for bacterial wilt of soybean. Based on the results of the experiments, it can be concluded that the Premax protector, as expected, ensures the preservation of bacteriophages to a greater extent than an aqueous solution, which allows longer storage of phage preparations in warehouses of agricultural producers. Comparison of the phage titer in seed washes shows that phage treatment together with seed inoculation can be effectively used, since this does not inactivate viral particles, but on the contrary, allows them to remain on the seeds longer in comparison with an aqueous solution. Bacteriophage Ayka is not able to translocate from the surface of seeds into sprouts, however, if the roots are damaged, as well as with sharp changes in soil moisture, it is able to translocate soybean plants from the soil, which shows the future prospects of conducting field experiments aimed at testing the efficiency of introducing bacteriophage into the soil during irrigation.

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