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ISOLATION OF BACTERIOPHAGES, CAPABLE TO LYSE SERRATIA MARCESCENS AND EVALUATION OF THEIR ACTIVITY ON ONION AND GERANIUM

In this study, phages active against S. marcescens, causative agent of onion decay, were isolated from plant material. One virus isolate was shown to accumulate in high titers and was denoted as phage S. This bacteriophage exhibited a hexagonal head and tail and was attributed to Myoviridae family. It was shown the ability of bacteriophage S to suppress the development of bacteriosis on geranium plants. Investigated virus isolate also inhibited rooting of onion scales. This work focused on a biological control approach to use bacteriophages for reducing bacterial pathogen populations and disease severity on plants.

Key words: bacteriophages, plant bacteria, phytopathology, phage therapy.

Introduction. Plant pathogenic bacteria cause many serious plant diseases throughout the world stimulating intensive research of their ecology, pathology and epidemiology. Bacterial rot (*Erwinia carotovora*) and vascular bacteriosis (*Xanthomonas campestris*) cause epidemics and lead to large harvest destruction, resulting in requirement of the import of cabbages, tomatoes and pepper [1]. Bacteria of genuses *Salmonella, Serratia, Enterobacter* and *Enterococcus,* more frequently happen in the cases of nocosomial infections, e.g. food poisonings and sepsises [2-5]. The last researches, however, prove that these human pathogenic bacterial species also have ability to colonize a wide spectrum of plants and cause the disease development. For the instance *Serratia marcescens*, common soil bacteria, was described as causative agent of soft rot of onion [6]. However, it should be noted that the majority of these experiments were performed under laboratory conditions, while the development of crop diseases, caused by these pathogens in the environment, is still poorly understood.

Bacteriophages, bacterial viruses, are widely present in the environment, wherever the host bacterium is expected to be found. Some bacteriophages are potentially useful agents in the control of plant pathogens. There is a wide array of potential candidate phage isolates; hence, investigations are needed to determine which isolates will likely be the most effective for application in bacterial control strategies. Our purpose was to search bacteriophages, specific to *S. marcescens* and to verify their activity against this bacterium on the plants of onion and pelargonium.

Materials and methods.

Bacterial strain. Studies were carried out on bacterial culture *Serratia marcescens* IMBG291 [5], generously provided by colleagues from the laboratory of microbial ecology, Institute of Molecular Biology and Genetics (National Academy of Sciences of Ukraine). Working with phages we used an overnight culture of bacteria, in which bacteria was in exponential phase of growth. The concentration of bacteria cell culture was 10^8-10^9 c.f.u./ml. Bacteria was cultivated on plate count agar or in PC-broth. Incubation temperature was 25 ^{O}C.

Bacteriophage isolation. In order to isolate bacteriophages the samples of plant displaying the symptoms of bacteriosis were used. To amplify putative bacteriophages in samples enrichment method was applied. Investigated samples were centrifuged and handled by chloroform. The objects of research became phage, isolated from samples of tomatoes with symptoms of rot processes.

Spot-titer assay. The samples or serial dilutions of samples were applied dropwise on the plates with seeded bacterial culture. Following 20 minutes they were kept at room temperature in order to samples diffused into agar medium. Then plates were overturned and incubated in a thermostat at 37^{0}C for 12 hours. After that the plates were analyzed for the presence of phages. Results were recorded as the reciprocal of the highest dilution at which clearing the lawn was evident.

Double agar layer method. 0.2 ml of overnight bacterial culture (10^8 c.f.u./ml) was put together with 2.5 ml of 0.7% agar (the temperature of agar was 46-49^{0}C). Then 1 ml of the studied sample was added. The resulting mixture was accumulated on the bottom layer of 1,4% agar [1]. According to the results of spot-test, the concentration of phage particles in a sample of carrot was very high, so we diluted the phage lyzate to the 10^{th} degree in order to get separated plaques. After exposure within 15 minutes at the room temperature, plates were inverted and incubated at 37^{0}C for 12 hours. After incubation all resulting plaques were counted. Separate phage plaques were then picked. Isolated bacteriophages were purified by serial propagation of single plaques and amplified.

Electron microscopy. Morphology of virions was investigated using the electron microscope. Formvar films placed on 400-mesh copper grids were dipped into sample for 2 min and contrasted in 2% uranyl acetate. The preparations were dried and viewed under the electron microscope at an instrumental magnification of 20,000.

Investigation of bacteriophages influence on the expression of pathogenic properties of the bacteria.

In laboratory conditions the infectious process was modeled with the onion inoculation with investigated bacteria *Serratia marcescens*. Onion scales were obtained from sterilized onion bulbs and incubated in plates on paper filter discs. The onion scales were scratched with sterile scalpel. Then 10mkl of phage mixture (titer 10^7) and bacterial suspension (concentration of cells 10^8 c.f.u./ml) were applied on the scales. For controlling the initiation of pathogenic process with bacteria a drop of bacterial suspension was applied on the scales of onion, placed in other plate. Onion scales with deposited drop of physiological solution served as a control of the experiment. All plates were placed in the incubator at 25^{o}C. For statistical significance of data obtained each experiment was conducted in three repetitions.

In order to research the potential of isolated bacteriophages as therapeutic agents another model system was used. The ability of bacteriophages to suppress the development of bacteriosis was investigated on geranium plants. For this purpose geranium leaves were infected with bacteria in two ways: injection into veins with subsequent incubation in water or adding bacterial suspension to water. We utilized different ways for the treatment of plant leaves with bacteriophage preparations. In the first group the leaves was inoculated in a vein with the suspension of bacterium and phage, in the second group plant leaves were inoculated with a bacterium into a vein and incubated in water supplemented with phages.

Results and discussion. Initial studies on the application of bacteriophage for control of pathogens require the identification and isolation of an appropriate phage from the multitude of phages that exist in the environment. Phages

specific for pathogenic *Serratia marcrscens* were isolated after plating the samples of rotten tomatoes. We have observed the formation of different morphology plagues on bacterial lawn. Investigated isolates resulted in formation of large and small negative colonies (fig. 1). Large colonies had round, smooth shape (d=5-6 mm), while small colonies were 1-2 mm in diameter and characterized with irregular shape.

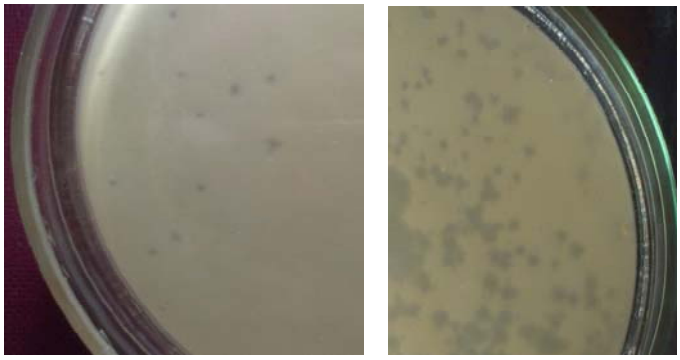

Fig.1. Bacteriophage plagues on *S.marcescens* lawn: isolate S (left), isolate L (right)

For subsequent research, we have chosen phages with small colonies because of their capability to accumulate in high titers compare to phages with large colonies. These phages were denoted as "S" in the experiment. To accumulate this isolate separate phage plagues were picked and transferred to sterile saline. Isolated bacteriophages were purified by serial propagation of single plaques. According to the results of spot-tests the titer of viruses after three passages was 10^{-7}. High titer lysates were routinely prepared from confluent lysis plates. Then we studied the morphological features of the selected bacteriophages using electron microscopy. The isolate S belongs to family of *Myoviridae* of order *Caudovirales* (morphotype A2, fig. 2).

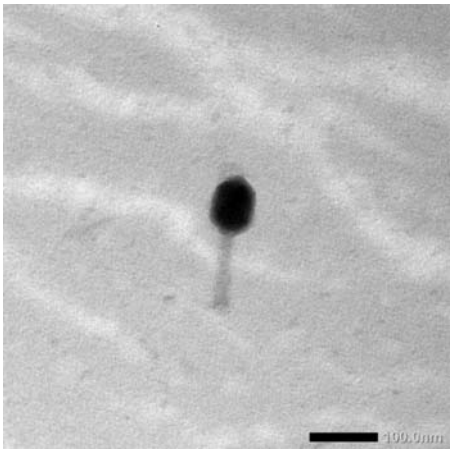

Fig.2. Transmission electron microscopy images of *S. marcescens* phage (S)

The next stage was to verify if the selected bacteriophage isolate S was active against *S. marcescens* in test-system on onion scales. In two days after inoculation we observed the development of soft rot on onion scales threated with bacterium and the absence of bacterial growth in the plate where phage was added.

Thus, our phage isolate was effective against *S. marcescens* in selected test system.

Fig. 3. Onion scales: inoculated with bacteria (left), inoculated with bacteria and phage (right)

In order to research the potential of isolated bacteriophages as therapeutic agents another model system was used. The ability of bacteriophages to suppress the development of bacteriosis was investigated on geranium plants. As a result, bacteria cased redness of leaves in five days after inoculation. The symptoms were the same in both variants of bacterial infection. Turning red was observed along the veins while the whole leaf plate remained green. In the site of bacteriophage inoculation we observed clean area. This suggested that bacteriophage repressed bacterial growth. However according to data obtained isolated phage was limited in the ability to spread through the plant tissue.

Conclusion. Consequently, the probed bacteriophage repressed the development of phytobacteriosis caused by *S. marcescens* on onion scales and geranium leaves. The use of bacteriophages to combat bacterial infections may help to solve the current problem of antibiotic resistance. For successful application of bacteriophages fundamental issues arising from the ecological dynamic of host, bacterium and phage should be investigated in detail.

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ВИДІЛЕННЯ БАКТЕРІОФАГІВ, ЗДАТНИХ ЛІЗУВАТИ SERRATIA MARCESCENS ТА ПЕРЕВІРКА ЇХ АКТИВНОСТІ НА ЦИБУЛІ ТА ПЕЛАРГОНІЇ

Бактеріофаги, активні проти S. marcescens, збудника гнилі цибулі, були виділені з рослинного матеріалу. Один із виділених ізолятів вірусів накопичувався у високих титрах і був позначений як бактеріофаг S. За морфологічними характеристиками (наявність голівки і хвостового відростку) даний вірус був віднесений до родини Myoviridae. Було показано здатність бактеріофагу S пригнічувати розвиток бактеріозу на рослинах пеларгонії. Досліджуваний ізолят також інгібував гнилісні процеси на лусочках цибулі. В даній роботі розглядається спосіб біологічного контролю фітопатогенів із застосуванням бактеріофагів для зниження чисельності бактеріальних популяцій і суворості прояву симптомів.

Ключові слова: бактеріофаги, фітопатогенні бактерії, фітопатологія, фаготерапія.

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ВЫДЕЛЕНИЕ БАКТЕРИОФАГОВ, СПОСОБНЫХ ЛИЗИРОВАТЬ SERRATIA MARCESCENS И ПРОВЕРКА ИХ АКТИВНОСТИ НА РАСТЕНИЯХ ЛУКА И ПЕЛАРГОНИИ

Бактериофаги, активные против S. marcescens, возбудителя гнили лука, были выделенны из растительного материала. Один из изолятов накапливался у высоких титрах и был обозначен как бактериофаг S. За морфологическими характеристиками (наличие головки и хвостового отростка) выделеный бактериофаг классифицирован как представитель семейства Myoviridae. Было показано способность бактериофага S подавлять развитие бактериоза на растениях пеларгонии. Исследуемый изолят вируса ингибировал также гнилостные процессы на чешуйках лука. В данной работе рассматривается способ биологического контроля фитопатогенов с применением бактериофагов для снижения чисельности бактериальных популяций и суровости проявления симптомов.

Ключевые слова: бактериофаги, фитопатогенные бактерии, фитопатология, фаготерапия.