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DETECTION OF *PROTEUS MIRABILIS* AND *ENTEROBACTER CLOACAE* IN TOMATTO AND PEPPER FRUITS AND ISOLATION OF THEIR BACTERIOPHAGES

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Samples of tomatoes and pepper with brown rotten lesions were collected during summer 2013. Bacterial associations were separated and investigated by microbiology methods. Bacteria Proteus mirabilis and Enterobacter cloacae were detected in rotten vegetables. Bacteriophages specific to identified bacteria were isolated by enrichment methods from the same samples. The morphology of bacteriophages was investigated using electron microscopy.

Key words: *Proteus mirabilis, Enterobacter cloacae, tomato, pepper, bacteriophages*

Plant pathogenic bacteria cause many serious diseases of plants throughout the world stimulating intensive research of their ecology, pathology and epidemiology. Leaf and fruit spots, blights, cankers, vascular wilts, rots and tumors are characteristic symptoms of bacteriosis. The list of the most important plant bacteria includes: Pseudomonas syringae, Ralstonia solanacearum, Agrobacterium tumefaciens, Xanthomonas sp., Erwinia amylovora, Xylella fastidiosa, Pectobacterium sp., etc [1].

Pathogenic bacteria utilize a number of mechanisms to cause diseases in plant hosts. As the plant bacterial pathogens are extracellular, they deploy a delivery of secreted virulence factors to interfere with host cell processes from outside plant cells. These include production of protein virulence factors (effectors), which are directly injected into host plant cell cytoplasm via a specialized type III secretion path, secretion of low molecular weight phytotoxins which are produced into apoplast, production of exopolysaccharides and cell wall degrading enzymes [2]. The mechanisms of pathogenicity of phytobacteria have sometimes shown surprising and unexpected similarity to those found in animal and human pathogens. Recent studies have shown that many plant pathogens have the capacity to colonize other hosts

outside of the plant kingdom, including insects, animals, and humans [3] and vice versa bacteria normally associated with animal hosts pathogenic are able to colonise plants and use them as alternative hosts. Many bacteria are described to exhibit cross-kingdom pathogenicity, where both humans and plants are potential hosts. However the investigations of cross-kingdom are still in relative infancy and there are far more questions than answers at present [4].

The majority of the research already published has focused on the enteric bacterial pathogens, there is no doubt that other human pathogens can also interact with plants. For the instance *Serratia marcescens*, common soil bacteria, was described as causative agent of soft rot of onion *(Allium cepa* L) [5]. Other enterobacteria soil inhabits such as *Enterobacter cloacae*, *Proteus mirabilis* were investigated as plant pathogens [6]. Most of these bacteria are harmless to plants and animals, but some strains are pathogenic for humans. *Pantoea agglomerans*, *Serratia marcescens*, *Enterobacter cloacae*, *Proteus mirabilis* – opportunistic nosocomial pathogens, in some cases lead to significant health problems [7]. The situation is complicated by widespread antibiotic resistance of this bacteria and their ability to switch to alternative sources of nutrition: from organic material in soil to organic material in plants and animals. These bacteria are polybiotrophic microorganisms and able to affect plants that serve as the sources of human infections.

In the last decade, fresh fruits and vegetables have been increasingly reported in association with foodborne illness [3, 8]. Many cases were associated with tomato and pepper. Despite this microbiological quality of tomatoes and pepper in Ukraine remains unclear.

Eradication of pathogenic microorganisms from crops is very important task and bacteriophages as natural bacterial antagonists have a great potential in development of antimicrobial control strategies. They have several benefits over chemicals currently used in agriculture. First of all, bacteriophages are highly specific, nontoxic for human and not harmful for normal microphlora of plants and soils [9].

Considering all mentioned above the purposes of this work were: (1) to isolate and identify the harmful bacteria from tomatoes and pepper grown up in Ukraine and (2) find bacteriophages, specific to isolated bacteria.

Material and methods. The content of brown rot was placed on different growth medium – LB, PDA, YDC, NA [10]. Separation of bacterial groups was performed by titration to 5 CFU/ml and plating on growth medium. To separate all bacterial isolates present in samples Petri dishes were incubated in a thermostat at a temperature of 27°C for 7 days. The morphological features of bacterial colonies were studied using stereoscopic microscope (Biomed MS-1 ZOOM). Farther on bacterial cells were stained according to Gram and examined on magnification x1600 (MICROmed XS5520) [11]. Type of respiration was established after tests on cytochromoxidase with Hugh-Leifson medium (main solution: peptone – 2, NaCl – 5, KH_2PO_4 – 0,3, agar – 3, bromothymol blue 0,1% solution – 3 ml, pH=7,1, glucose solution: 10 g glucose, 60 ml dH_2O) [12]. Diagnostic biochemical profiles of bacteria were investigated with test-systems “ENTERO-16” and “NEFERM-21”, Erba Lachema, Czech Republic. Advanced tests were carried out to determine the ability of isolated bacteria to hydrolyse gelatin by the method of gelatin columns (MPG: peptone – 10, NaCl – 5, meat extract – 3, gelatin – 10, agar – 20, bring to pH 7.0 with 10% solution of $NaHCO_3$) and the ability to restore nitrate in nitrate broth (meat extract – 3, peptone – 5, KNO_3 – 2, NaCl – 20, agar – 10, pH=7.0) with Kasatkin reagent (solution A: 0.1% solution of rivanol on distilled water, solution B: 12% solution of HCl) [10]. The sensitivity to antibiotics was determined by bacteriological analyzer "VITEK-2" [13]. Phytopathogenic properties of isolates was conducted on potato slices [14], young plants of *Nicotiana tabacum* var. *“Samsun”* after inoculation by bacteria in leaf and stem streaks in concentration 10^7 CFU/ml [15].

In order to isolate bacteriophages the same samples after surface sterilization were used. To amplify bacteriophages in samples enrichment method was applied. For this purpose the content of brown rot lesions was loaded into liquid LB broth and incubated for 48 hours at 27°C. After incubation LB broth was subjected to low-speed centrifugation (5000 r/m, 25 min), supernatant was mixed with chloroform to remove bacteria. The samples were plated on a bacterial lawn by agar overlay method [16]. Separate phage plagues were than picked and transferred to sterile saline (1 ml). Isolated bacteriophages were purified by serial propagation of single plaques.

High titer lysates were routinely prepared from confluent lysis plates by adding 10 ml of saline to 10 plates. After 30 min, the soft-agar layers were scraped off with a bent glass rod. The crude lysates were clarified by low speed centrifugation at 15.000g for 15 min. The supernatant was centrifuged at 98000 g for 120 min (centrifuge UCP-65, RCP-50 rotor) [17]. The pellet was suspended in saline.

For bacteriophage staining, phage solution was deposited on formvar coated copper grid for two minutes and stained by 2% (w/v) uranyl acetate, pH 4 - 4.5. The solution was drained through filter paper and phage particles were observed through transmission electron microscopy (JEOL 1400, instrumental magnification of 40.000-90.000) [18].

Results and discussion. A total of 24 tomato (*Solanum lycopersicum* L) and 12 pepper (*Capsicum anuum* L) fruits were taken from agrocenosis of Kirovogrdska and Khersonska regions, respectively. The symptoms of soft brown rot, tissue maceration, extraction of exudates were observed on tomato and pepper fruits (fig 1). Taking into account that plants can be invaded with wide range of bacteria including enterobacteria the samples were plated on different types of media: LB (lytic broth) – common for many enterobacteria, PDA (potato dextrose agar) – common for most phytopathogenic bacteria, YDC (yeasts dextrose-carbonate agar) – medium for cultivation of bacteria from genus *Xanthomonas*, MPA (meat-peptone agar) – common for many bacteria, including soil and pathogenic bacteria.

On YDC medium most of isolates formed mucous, exopolysaccharide-rich colonies. After dilution to 5 CFU/ml and plating on LB, PDA, MPA mediums isolated bacterial colonies were easy separated into different bacterial isolates according to their morphological properties. Two of them – II (tomato) and XXIV (pepper) demonstrated rapid growth on MPA, PDA, LB-media that was not typical for plant pathogenic bacteria. These isolates were selected for further investigation as putative enterobacteria.

Figure 1. Naturally infected tomato fruits showing symptoms of bacterial infection.

Isolate II produced large (d=7 mm), round, milky-green colonies with a wavy edge, convex profile, smooth and shiny surface. After 24 h incubation of isolate II we observed the formation of concentric rings departing from the main colony that suggested the bacterial mobility (fig. 2).

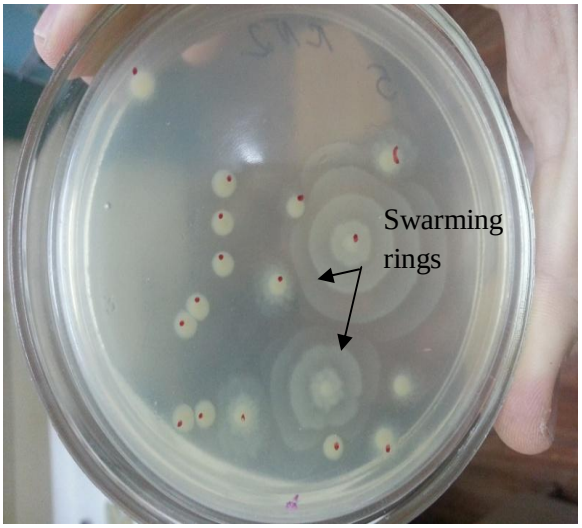

Figure 2. Colonies of bacterial isolate II on MPA, swarming from central colony. Incubation time – 24 h. Bacterial colonies recovered from tomato fruits

Isolate XXIV formed large (d = 7 mm), round, greenish colonies with a raised profile, smooth edge and smooth and shiny surface.

The bacterial smears were stained according to Gram and studied using light microscope. Both isolates were shown to be Gram-negative, rod-shaped bacteria, 1-4 μm in length with hundreds of cilia.

In Hugh-Leifson medium bacterial grew changed color of medium and produced gas bubbles in aerobic and anaerobic conditions, indicating that both isolates were

able to ferment glucose. A similar phenomenon is typical for facultative anaerobic bacteria. Test on β-galactosidase was positive for isolate XXIV.

Investigations of bacterial biochemical profiles were conducted using test-systems, which are mainly used for the detection of clinical strains, but majority biochemical markers are common for phytopathogenic and pathogenic for human bacteria.

The results of bacterial identification are presented in tab 1. Isolate II was classified as *Proteus mirabilis*, isolate XXIV – *Enterobacter cloacae* (tab. 1). The comparison of the data with Bergey's Manual of Systematic Bacteriology [19] confirmed the results of biochemical analysis.

***1*. Biochemical profiles of bacterial isolates II (in comparison with *Proteus mirabilis*) and XXIV (in comparison with *Enterobacter cloacae*)**

[1]Biochemical marker	OXI	ONP	H_2S	LYS	IND	ORN	URE	PHE	ESL	SCI	MAL	INO	ADO	SUC	SOR	TRE	MAN	GEL	NIT
Isolate II	-	-	+	-	-	+	+	+	-	+	-	-	-	-	-	+	-	-	+
*Proteus mirabilis**	-	-	+	-	-	+	+	+	-	+	-	-	-	-	-	+	-	-	+
Isolate XXIV	-	+	-	-	-	-	-	-	+	+	+	-	-	+	+	+	+	-	+
*Enterobacter cloacae**	-	+	-	-	-	-	-	-	+	+	+	-	-	+	+	+	+	-	+

A high percentage of overlap between clinical strains and isolates obtained from rotten tomatoes may indicate widespread of opportunistic pathogens in agrocenoses.

The results obtained after verification bacteria on antibiotic resistance and making estimates about its further progression analyzer "Vitek-2" showed, that both bacteria are resistant to some antibiotics. The resistance to amoxicillin, cefalexin, cefpodoxime, enrofloxacin was obsearved in *Enterobacter cloacae. Proteus mirabilis* was shown to be resistant to enrofloxacin, nitrofurantoin, tetracycline. *Enterobacter*

1 OXI – cytochromoxydase, ONP – β-galactosidase, H_2S - hydrogen sulfide production, LYS – decarboxylase of lysine, IND – indol production, ORN – decarboxylase of ornithine, URE – urea hydrolysis, PHE – phenilalaninedeaminase, ESL – aesculine hydrolysis, SCI – citrate utilization, MAL – malonate utilization, INO – acid from myo-inositole, ADO – acid from adonitol, SUC – acid from saccharose, SOR – acid from sorbitol, TRE – acid from trehalose, MAN – acid from mannitol, GEL – gelatine hydrolysis (22 °C), NIT - nitrate reduction.

* Data from Bergey's Manual of Systematic Bacteriology.

cloacae also displyed low sensitivity rate to nitrofuran that indicate the development of resistance. The results of bacteral susceptibility to antibiotics are presented in the table 2.

2. Antibiotic sensitivity profiles of isolated bacteria

Antibiotic	Bacteria species			
	Enterobacter cloaceae		*Proteus mirabilis*	
	MIC	Reaction	MIC	Reaction
Amikacin	≤2	S	≤2	S
Amoxicillin	≥32	R	≤2	S
Ampicillin	-	-	≤2	S
Cefalexin	≥64	R	16	S
Cefpirome	≤1	S	≤1	S
Cefpodoxime	0,5	R	≤0,25	S
Celtiofur	≤1	S	≤1	S
Chloramphenicol	≤2	S	-	-
Enrofloxacin	≤0,12	R	≤0,12	R
ESBL	-	-	-	-
Gentamicin	≤1	S	≤1	S
Imipenem	≤2	S	-	-
Marbofloxacin	≤0,5	S	≤0,5	S
Nitrofurantoin	64	I	256	R
Piperacin	≤4	S	≤4	S
Polymyxin B	-	-	-	-
Rifampicin	-	-	-	-
Tetracycline	≤1	S	≥16	R
Tobramycin	≤1	S	≤1	S
Trimethoprim	≤20	S	≤20	S

According to data obtained these bateria can serve as source of antibiotic resistance factors. Subsequent horizontal gene transfer with other plant and human

pathogenic bacteria in the environment can lead to emergency new multidrug-resistant bacteria.

Determination of pathogenic properties was performed on indicator plants (*Nicotiana tabacum* var. *"Samsun"*) and potato slices. Both bacteria caused the formation of necrotic lesions on tobacco leaves after inoculation into the lateral streak (fig. 3). The treatment of potato cubes with bacterial suspension did not cause tissue maceration, indicating a lack of pectinase and amylase, common virulence factors of plant pathogenic bacteria.

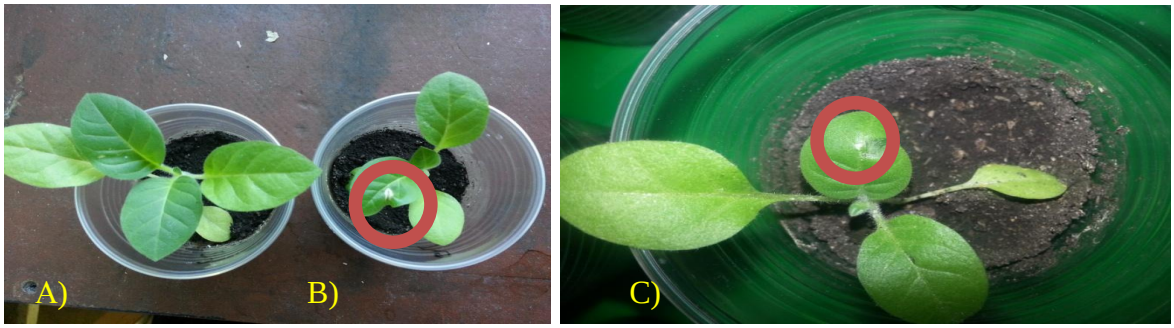

Figure 3. Formation of necrotic lesions on the leaves of tobacco plants after bacterial inoculation into lateral leave streak.

A – control plant, B – inoculation by isolate II (*Proteus mirabilis*) and C – by isolate XXIV(*Enterobacter cloacae*). Formation of necrotic lesions occurs after 12 h.

In order to develope a biocontrol approach to manage isolated bacteria we attempted isolation specific bacteriophages from vegetable fruits. As a result two bacteriophages, specific to *Proteus mirabilis*, were isolated. Bacteriophages, named φ*Prm*1 and φ*Prm*2 produced small plaques, d<1 mm. Data, obtained with an electron microscope for phage isolates indicated that viruses were members of family *Myoviridae* (fig 4). Isolate φ*Prm*1 had icosahedral head 80X80±4 nm and tail 90±5 nm, whereas isolate φ*Prm*2 consisted of head 110X110±6 nm in diameter and elongated tail 300±10 nm in length.

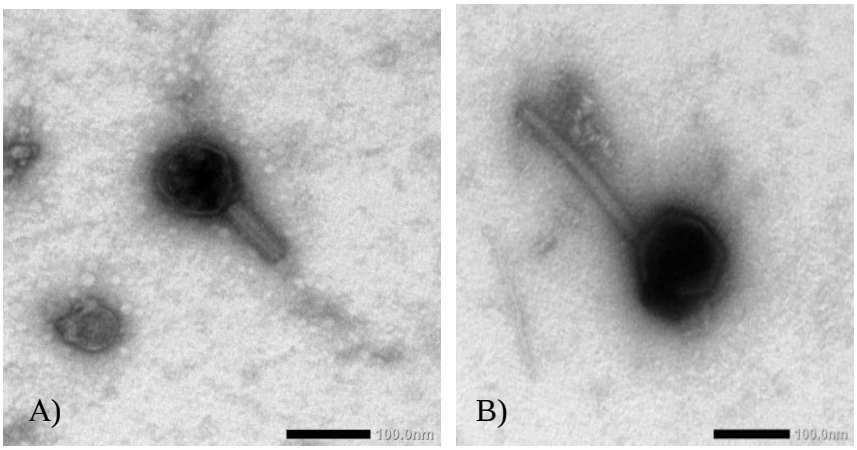

Figure 4. Morphology of the bacteriophages isolates φ*Prm*1 (A) and φ*Prm*2 (B). Both phages belong to *Myoviridae* family, morphotype A1.

It were also isolated 3 bacteriophages, specific to *Enterobacter cloacae*. Isolates formed plaques with different morphological properties: isolate φ*Entc*1 formed giant plaques 5-7 mm in diameter with concentric, ring-like circles around central zone. Phages isolates φ*Entc*2 and φ*Entc*3 produced middle (3 mm) and small (1 mm) plaques. Data, obtained with an electron microscope for phage isolates indicate that viruses belong to 3 distinct families – isolate φ*Entc*1 is a member of *Podoviridae*, morhpotype C3, it has an elongated head (80X30±4 nm) and shot tail (15±3 nm); isolate φ*Entc*2 is a member of *Inoviridae* (flexible filamentous bacteriophages about 600±30 nm in length, morphotype F); isolate φ*Entc*3 is a member of *Siphoviridae family*, type B1, head size 50X50±4 nm, tail – 100 nm±5 (fig. 5).

Isolated bacteriophages did not interact with other bacteria isolated from samples of tomatoes. Isolated phage did not interact also with related to the host species bacteria, such as *Escherichia coli*, *Pectobacterium carotovorum*, *Pectobacterium amilovorum*, *Serratia marcescens*, *Pantoea agglomerans*. Such specificity formed between virus and host only after prolonged coexistence populations of phages and bacteria on farmland.

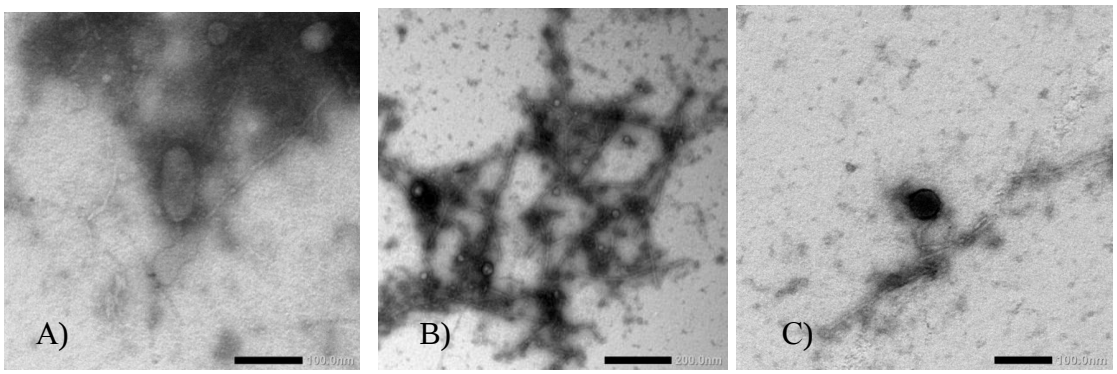

Figure 5. Bacteriophages morphology, obtained after TEM: A) member of *Podoviridae* family, morphotype C3; B) member of *Inoviridae* family, morphotype F; member of *Siphoviridae* family, morphotype B1.

Detected microorganisms could be introduced in agrocenoses with organic fertilizers, such as humus. Probably violation of crop rotation led to introduction of opportunistic microorganisms into new ecological niches and invasion of plants such as tomatoes. Lack of enzymes breaking down cellulose indicates that the bacteria are unable to digest plant matherial and require the presence of phytopathogenic bacteria capable to destruct plant cells and release nutrients for *Proteus mirabilis* and *Enterobacter cloacae*. Meanwhile, these features do not reduce the risk for a person who consume contaminated vegetables as fresh salads and juices. The existence of such bacteria in agrocenoses, their distribution among plant varieties necessitate revision old strategies to control dissemination of opportunistic bacteria in crops.

Conclusion

As the results were isolated 24 isolates of bacteria from tomato fruit with brown rot. Specific broth was found, biochemical profiles of isolated bacteria were identified. Taxonomic position of 2 isolates was established, thus isolate II is *Proteus mirabilis*, isolate XXIV – *Enterobacter cloacae*. Bacteriophages, specific to the pathogenic microorganism isolated and examined by the method of electron microscopy. The selected isolates belonging to the 4 families: *Myoviridae*, *Siphoviridae*, *Podoviridae* and *Inoviridae*.

REFERENCES

1. Patyka V. P. Phytopathogenic bacteria in the system of modern agriculture / V. P. Patyka, L. A. Pasichnyk // Мікробіологічний журнал. – 2014. – Т. 76, №1. – С. 21-26.

2. Prasannath K. Pathogenicity and Virulence Factors of Phytobacteria K. Prasannath // Scholars Academic Journal of Biosciences. – 2013. – Vol. 1, Is. 1. – P. 24-33.

3. Heather L. T. Plants as a Habitat for Beneficial and/or Human Pathogenic Bacteria / Heather L. T., Triplett E. W. // Annu. Rev. Phytopathol. – 2008. – Vol. 46. – P. 53-73.

4. Kirzinger M. W.B. Insights into Cross-Kingdom Plant Pathogenic Bacteria / M. W. Kirzinger, B. G. Nadarasah, J. Stavrinides // Genes. – 2011. – Vol. 2. – P. 980-997.

5. A mobile genetic element in *Serratia marcescens*, a causative agent of onion disease / L. P. Ovcharenko [et al]. // Biopolymers and Cell. – 2010. – Vol. 26, N 4. – P. 279-285.

6. Janda J. M. The Enterobacteria, 2nd ed.// J. M.Janda, S. L. Abbott– Washington D.C.: ASM press, 2006. – 411 p.

7. Guidelines for Prevention and Treatment of Opportunistic Infections in HIV-Infected Adults and Adolescents / J. E. Kaplan [et al]. // Recommendations from CDC, the National Institutes of Health, and the HIV Medicine Association of the Infectious Diseases Society of America. – 2009. - № 58, – P. 1-198.

8. Bacteria Associated with Foodborne Diseases / R. S. Flowers [et al]. // Food Technology. – 2004. – Vol. 54, Is. 7. – P. 1-25.

9. Bacteriophages for Plant Disease Control / J. B. Jones [et al]. // Annu. Rev. Phytopathol. – 2007. – Vol. 45. – P 245–262.

10. Atlas R. M. The Handbook of Microbiological Media for the Examination of Food, 2nd ed / R. M. Atlas– Boca-Raton: CRC Press, 2006. – 464 p.

11. Snyder J.W. Handbook of Media for Clinical Microbiology. / J. W. Snyder, R. M. Atlas– Boca-Raton: CRC Press, 2006. – P. 347.

12. Радченко О. С. Фізико-біохімічні властивості мікроорганізмів та методи їх вивчення / О. С. Радченко– Київ: ТОВ «Аграр Медіа Груп», 2012. – 211 с.

13. Evaluation of the New VITEK 2 Extended-Spectrum Beta-Lactamase (ESBL) Test for Rapid Detection of ESBL Production in *Enterobacteriaceae* Isolates / T. Spanu [et al]. // J. Clin. Microbiol. – 2006. – Vol. 44, Is. 9. – P. 3257–3262.

14. Метлицкий Л. В. Пектолитические ферменты / Л. В. Метлицкий, К. В. Васильева в Биохимия иммунитета и покоя растений. – М.: Наука, 1969. – 358 с.

15. Klement Z. Hypersensitive reaction induced by phytopathogenic bacteria in the tobacco leaf / Z. Klement, G. Farkas, L. Lovrekovich // Phytopathology. – 1964. – Vol. 54. – P. 474-477.

16. Carlson K. Bacteriophages: Biology and Applications. Appendix: Working with bacteriophages: Common techniques and methodological appproaches / K. Carlson, E. Kutter, A. Sulakvelidze – Boca Raton: CRC Press, 2005. – PP. 437-494.

17. Остерман Л. А. Методы исследования белков и нуклеиновых кислот: электрофорез и ультрацентрифугирование / Л. А. Остерман– М.: Наука, 1981. – 288с.

18. Миронов А.А. Методы электронной микроскопии в биологии и медицине: Методологическое руководство / А. А. Миронов, Я. Ю. Комиссарчик, В. А. Миронов– СПб.: Наука, 1994. – 400 с.

19. Хоулт Д. Определитель бактерий Берджи: Т1 / Д. Хоулт [и др]. – М.: Мир, 1997. – 800 с.

ДЕТЕКЦІЯ БАКТЕРІЙ *PROTEUS MIRABILIS* ТА *ENTEROBACTER CLOACAE* В ПЛОДАХ ТОМАТІВ ТА ОВОЧЕВОМУ ПЕРЦІ І ВИДІЛЕННЯ ЇХНІХ БАКТЕРІОФАГІВ

А. В. Харіна, С. А. Заіка, Ю.М. Юмина, П. П. Зелена, Н. О. Корнієнко, Ю. А. Косенко, В. П. Поліщук

Зразки томатів (Solanum lycopersicum L) і перцю овочевого (Capsicum annuit L), які мали симптоми у вигляді плям бурого загнивання були відібрані влітку 2013 року. Асоціації бактерій розділили на окремі культури і дослідили за допомогою мікробіологічних методів. Визначено, що у зразках уражених плодів є бактерюфаги, здатні лізувати ізольовані бактерй, їх виділили методом збагачення. Морфологічні особливоспи вірусів досліджено за допомогою трансмісійного електронного мікроскопу. Ізольовані бактерюфаги можуть бути розглянуті у якості фаготерапевтичних агентів проти виділених культур бактерій.

Ключові слова: *Proteus mirabilis, Enterobacter cloacae, томати, перець овочевий. бактеріофаги*

ДЕТЕКЦИЯ БАКТЕРИЙ PROTEUS MLRABILIS И ENTEROBACTER CLOACAE В ПЛОДАХ ТОМАТОВ И ОВОЩНОМ ПЕРЦЕ И ВЫДЕЛЕНИЕ ИХ БАКТЕРИОФАГОВ

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Образцы томатов (Solanum lycopersicum L) и перца овощного (Capsicum annuit L), которые имели симптомы в виде пятен бурого загнивания были отобраны летом 2013 года. Ассоциации бактерий разделили на отдельные культуры и иследовали с помощью микробиологических методов. Определено, что в образцах пораженных плодов является бактерюфагы, способны лизировать изолированные бактерий, их выделили методом обогащения. Морфологические особености вирусов исследованы с помощью трансмиссионного электронного микроскопа. Изолированые бактерюфагы могут быть рассмотрены в качестве фаготерапевтичних агентов против выделенных культур бактерий.

Ключевые слова: *Proteus mirabilis, Enterobacter cloacae, томаты, перец овощной. бактериофаги*