Methods of identification of phytopathogenic bacteria *Pectobacterium carotovorum subsp. carotovorum*

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Abstract. Bacterial infections are a global problem. The most common pathogens of infections among gram-negative bacteria are representatives of the *Enterobacteriaceae* family. *Pectobacterium* are gram-negative phytopathogenic bacteria belonging to the *Enterobacteriaceae* family.The purpose of the study is to develop methods for the identification of phytopathogenic bacteria. At the capacity of test components of developed identification algorithm, we used the data presented in the reference «Bergey's Manual of Systematics of Archaea and Bacteria». Model microorganisms for selection of research parameters and bacteriological tests were reference strains of *Pectobacterium carotovorum* B-3455 obtained from the All-Russian collection of microorganisms and *Pectobacterium carotovorum* 333 from the collection of NRCEM Museum of FSBEI HE Ulyanovsk SAU named after P.A. Stolypin. From 50 samples of phytosanitary control and environmental objects, 5 strains were classified as *Pectobacterium carotovorum spp. carotovorum*.

1 Introduction

Phytopathogenic bacteria *Pectobacterium spp.* of the family *Pectobacteriaceae* gram-negative, pectinolytic, necrotrophic bacterial pathogens of plants that cause soft rot, damping out and stem wilt leading to significant economic losses worldwide [1-5]. Bacteria *Pectobacterium* produce enzymes destroying the cell wall that allow them to penetrate and macerate plant tissue [6,7]. Pectobacterium carotovorum subsp. carotovorum belongs to the family *Enterobacteriaceae*, which causes mild rot and infections in many plants around the world, resulting in significant economic losses [8-13]. Gram-negative phytopathogens *Pectobacterium carotovorum subsp. carotovorum*, which produce and secrete enzymes, destroy the cell wall of plants, the action of which results in rotting and decomposition of their hosts in the field and during storage [14].

Soft rot caused by Pectobacterium species is a destructive plant disease and the spread of disease is difficult to control [15-17]. Currently, there is no effective antibacterial treatment against *Pectobacterium carotovorum subsp. carotovorum* [18]. The disease development depends on local climatic conditions [19]. Spread by water, insects, or tools

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[20-21]. Soft rot caused by P. carotovorum, is the most widespread potato disease. Bacterial soft rot of potato is one of the most significant factors limiting production of potatoes worldwide [22-23].

Phytopathogenic bacteria referred to *Pectobacterium* (soft rot *Pectobacteriaceae*) are in the focus of agricultural microbiology due to their diversity, significant negative impact on the production of potatoes and vegetables, as well as the prospects for bacteriophages application for disease control [24].

Soft rot was observed on potatoes and bell peppers with typical symptoms of soft rot and damping out. The bacteria were isolated from infected fruits, tubers, and stems and initial identification was based on morphological, biochemical, physiological, and pathogenicity tests. Isolates were aggressive, causing discoloration of plant tissues, damage to leaves, stems, wilting and death of plants. Inoculation of bell pepper fruits and potato tuber sections in various concentrations led to maceration of tissues and complete fruit rot [25-27]. *Pectobacterium* is a complex taxon of strains with various characteristics. The characteristic of Pectobacterium species allows to analyse variety of pectinolytic bacteria, which can support strategies to control bacterial diseases in plants [28].

Isolation, detection, identification, and characteristic of *Pectobacterium* species is achieved by using selective medium containing pectate. The pathogenicity test is performed by inoculating the bacteria into potato tubers. The biochemical and physiological characteristics of the strains were carried out using tests that are commonly used to differentiate *Pectobacterium* subspecies. Bacterial strains have a round shape, smooth edges, convex colonies with a diameter of 1 to 3 mm with a smooth texture, have a shiny yellowish-cream color. Gram-negative, catalase-positive and oxidase-negative [29]. Controlling pectolytic bacteria (soft rot) is still a difficult task in potato production around the world. Consumer demand for more natural and less toxic alternative post harvest disinfectants has led the industry to develop a variety of fresh food disinfectants and methods to prevent soft potato rot [30].

The aim of the study is to develop a diagnostic method for the phytopathogenic bacterium Pectobacterium carotovorum.

2 Materials and methods

Experimental works were carried out in FSBEI HE "Ulyanovsk SAU named after P.A. Stolypin" in 2016-2019. As test components of the developed identification algorithm, we used the data presented in the reference book "Bergey's Manual of Systematics of Archaea and Bacteria" (2015) [31]. The study of biological properties was carried out using classical methods [32-34]. Model microorganisms for selection of research parameters and bacteriological tests were reference strains of *Pectobacterium carotovorum* B-3455 obtained from the all-Russian collection of microorganisms and *Pectobacterium carotovorum* 333 from the Museum collection SRICMB FSBEI HE Ulyanovsk Sau named after P.A. Stolypin. In the research we used 123 samples from the objects of phytosanitary control and the environment.

3 Results and Discussion

For isolation and bacteriological identification of microorganisms, it was important to choose a selective medium, the characteristic growth on which could become the primary differential indicator. For this purpose, bacterial growth was analyzed on the following media: SL-CVPAG366 (figure 1), potato agar with 2,3,5-triphenyltetrazolium chloride (figure 2). Dark red colonies with a narrow colorless edge are formed on potato agar with

2,3,5 – triphenyltetrazolium chloride (TTX). For selecting the most promising medium for isolation scheme of Pectobacterium carotovorum spp. carotovorum, it was experimentally established that the medium with pectate SL-CVPAG-366 gives the best results, as the cultures form cup shaped bowls, causing deliquation of pectate. According yo the research data of M. C. M. Perombelon, J. M. Van Der Wolf (2002) [35] during the formation of cavities on the medium CVP we can presumably identify as the most common soft rot bacteria P. carotovorum. On the medium pectate SL-CVPAG-36, P. carotovorum form submerged grayish-light lilac colonies in cup-shaped depressions.

The cultivation temperature is 28ºC. After 24 hours, turbidity of the medium is formed on the meat-peptone broth (figure 3). After cultivation at a temperature of 28ºC, smooth, grayish-white, raised, shiny colonies of rounded shape with smooth edges form on meat-peptone agar after 24 hours.

The tinctorial and morphological properties of the strains were studied by Gram staining in smears from meat-peptone agar. It was found that the studied strains are gram-negative, short sticks with rounded edges. The cells are arranged singly, in pairs, in short chains. Spores and capsules do not form (figure 4).

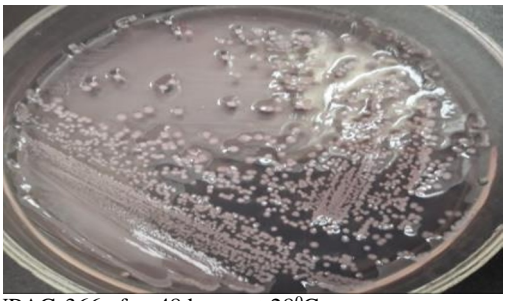

Fig. 1. Growth by SL-CVPAG-366 after 48 hours at 28^0C

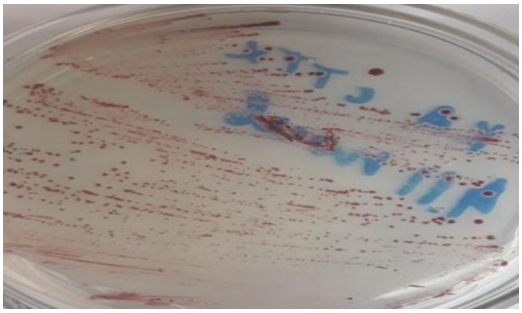

Fig. 2. Growth on potato agar from 2,3,5 TTH after 48h at 28C

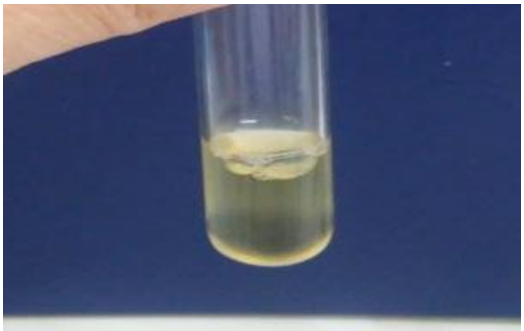

Fig. 3. Growth on meat-peptone broth after 24h at 28C

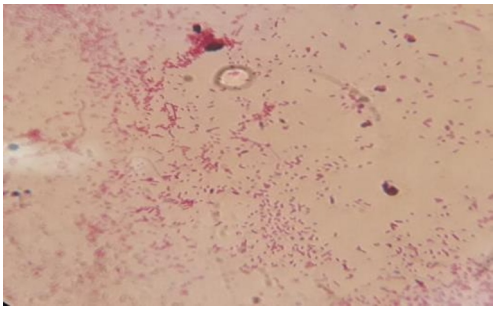

Fig. 4. Coloration of *P. carotovorum subsp. carotovorum* strain 3455 by Gram (magnification x 900 times)

The results of studies on biological properties of isolated bacteria do not differ from the data of reference strains Pectobacterium carotovorum B-3455 and Pectobacterium carotovorum 333 (Table 1).

Table 1. The main properties of the bacteria *Pectobacterium carotovorum subsp. carotovorum*.

Indicators	Model microorganisms		Research results of 5 strains	
			+	-
Potato agar with 2,3,5-TTX	+	+	5	
Cromogenesis on King B	-	-		5
Growth on medium SL CVP_{AG366}	+	+	5	
Phytopathogenicity control	+	+	5	
Coloring by Gramm	-	-		5
Ornithine decarboxilase	-	-		5
Indole	-	-		5
Mobility	+	+	5	
O/F test	f	f	5f	
Growth at 40^0C	-	-		5
Susceptibility to erythromycin	-	-		5
Oxidase	-	-		5
Catalase	+	+	5	
Lecithinase	-	-		5
Gelatinase	+	+	5	
Growth at 5% NaCL	-	-		5
Growth at 37^0C	+	+	5	
Simmons citrate	+	+	5	
Caseinase	+	+	5	
Methyl red	+	+	5	
Sorbitol	-	-		5
Sucrose	+	+	5	
Lactose	+	+	5	
Raffinose	+	+	5	
Rhamnose	+	+	5	
Maltose	-	-		5
Trehalose	+	+	5	

Developent of fast, low-cost, sensitive and specific approaches available to any production laboratories to pathogens and their detection is essential. Detection approaches involving bacteriophages as recognition elements get great attention due to their high degree of specificity, accuracy, and reduced analysis time [36]. For this purpose, we developed a scheme for accelerated identification of *Pectobacterium carotovorum* bacteria using strict specificity of selected phages. Isolated phages PCC–1 UlGAU, PCC–37 UlGAU have high lytic activity 10^{-5} an 10^{-8} by Appelman,1,0 x 10^8 and 3,0 x 10^9 corpuscles in 1 ml by Gratia.

Identification. A daily culture was applied to cups with meat-peptone agar, distributed over the surface and dried for 20 minutes. Then the cup was divided into sectors and drops of bacteriophages RS-1 Ulgu and RDS-37 Ulgu were applied with a sterile pipette. A control was applied to the third sector - a sterile meat-peptone broth and tilted so that the drops flowed down. After drying the dripping drops, the cups were incubated for 16-18 hours at 28oC. The lysis zone shows that the studied strain belongs to P. carotovorum subsp. carotovorum. The absence of lysis was considered a negative result

Phage identification of isolated strains as P. carotovorum spp. carotovorum was proved by obtained results of biochemical properties of bacteria.

4 Conclusion

Data on the main properties of Pectobacterium carotovorum subsp. carotovorum bacteria are presented. A method for diagnosing the bacterium Pectobacterium carotovorum is proposed.

The conducted studies prove the possibility of using a scheme for the identification of the bacterium P. carotovorum spp. carotovorum using a bacteriophage biopreparation consisting of bacteriophages RCC - 1 UlGAU and RCC - 37 UlGAU, which allows several times to reduce the time spent on research.

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