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Biological Properties of Phytopathogenic Bacteria *Pseudomonas syringae,* Isolated from Sugar Beet

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Pseudomonas syringae **bacteria were isolated and identified from the sugar beet leaves with the characteristics of destruction. Their biological properties were determined and compared with collection strains of phytopathogenic bacteria that cause bacterial disease of this culture. The isolated strains caused the necrotic dark spot formation on the leaves upon artificial inoculation of sugar beet plants, which gradually increased in size, while the tissues inside of the spots got dry and fell out. We demonstrated that the lipids of bacteria cells, isolated from infected leaves of sugar beet, include fatty acids, which are typical for Pseudomonas syringae according to quantitative and qualitative composition. The studied strains showed serological relationship with antiserum against strains- pathovars P. syringae of various serogroups. The high titer of the investigated strains in agglutination reaction with antiserums against representatives of IV serogroup indicates their affiliation to the serogroup IV.**

Keywords: Bacterial diseases, Isolates, properties, Identification, *Pseudomonas syringae.*

Crop roots of sugar beet by their anatomical and chemical properties are extremely favorable substrate for the development of numerous microorganisms of parasitic and saprophytic nature. However, a number of diseases of different etiology affect also the aboveground plant organs (Sabluk *et al*., 2005).

Leaf spot and stripe diseases are widespread among bacterial diseases of the leaf apparatus of sugar beet, that are caused by bacteria of the genus *Bacillus* and *Pseudomonas*, which can be found in the soil on the plant debris or exist on the plants as epiphytes (Gvozdiak *et al*., 2011; Pasichnyk L.A. *et al*., 2014).

The problems regarding the crop preservation and reduction of the negative impact of sugar beet diseases can not be solved without clear information about the characteristics of pathogens that cause them. Moreover, information about species composition of pathogens provides an opportunity to substantiate and develop an effective plant protection measurement system. That is why the purpose of our study was the isolation of phytopathogenic bacteria from sugar beet leaves with the characteristics of destruction, determination of their morphological and cultural-biochemical properties and their identification.

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MATERIALS AND METHODS

The object of the research were bacteria isolates, allocated from sugar beet leaves and sampled on the test plots in Uladovo – Lyulinets Experimental Breeding station of the Institute of Bioenergy Crops and Sugar Beet of NAAS of Ukraine. For comparative analysis the phytopathogenic bacteria that cause sugar beet diseases were used, namely: *Pseudomonas syringae* van Hall 1902 strains 7923, 7921 0nd *Pseudomonas syringae* pv. *aptata* (Brown & Jamieson, 1913) Young, Dye & Wilkie 1978 strains 8544 and 8545, which are stored in the collection of alive cultures in Phytopathogenic Bacteria Department in the D.K. Zabolotny Institute of Microbiology and Virology of NAS of Ukraine.

For the isolation of bacteria from the washed leaves the pieces which cover a part of the affected and healthy tissue were cut out, then pounded in a mortar with 0,5 ml of milliQ water and used for bacteria growing on the surface of potato agar (PA). Bacteria were grown at the temperature of 28°C during 5 days.

The ability to induce the hypersensitivity reaction was determined by introducing a suspension of bacterial cells under the epidermis of tobacco leaves by the Klement's method (Klement *et al*., 1990). Pathogenic properties of the studied bacteria were analyzed by artificial infection of sugar beets and beans (Beltukova *et al*., 1968).

Morphological, cultural and biochemical characteristics of allocated isolates were determined using classical methods (Beltukova *et al*., 1968; Gerhard, 1983). In order to determine the spectrum of carbohydrate fermentation and the presence of certain enzymes, the test systems API 20E (*bioMérieux*, France) were used. Determination of fatty acid (FA) composition of common cellular lipids was conducted according to the Brian's methods (Brian and Gardner, 1968). Separation of fatty acid methyl esters (FAME) was carried out using chromatography/mass selective detector system Agilent 6890N/5973 inert. Identification of allocated bacterial isolates was done by comparing their properties with collection strains and characteristics described in the Guide to bacterial determination (Brenneu *et al*., 2005). In addition, serological properties were determined by the agglutination reaction with application of antisera against strains allocated from cereals and sugar beet. According to the scheme of serogrouping of bacteria (Pastushenko and Simonovych, 1979), they belong to different serological groups: *Pseudomonas syringae* pv. *syringae* van Hall 1902 strain UKM - 1027 – serological group; *P. syringae* pv. *atrofaciens* strain K-1025 – serological group; *P. syringae* pv. *atrofaciens* strain UK" B- 1011) – V serological group; *P. syringae* pv. *atrofaciens* strain UK" B - 1115 – VI serological group; *Pseudomonas syringae* pv. *aptata* strains 8544 and 8545 - V serological group.

Live cultures of bacteria served as antigens. For experiments the working dilutions of antiserum in physiological solution were prepared with titers from 1:100 to 1:51200. Agglutination reaction was considered positive if flocculent precipitate was formed in the tubes and supernatant fluid became transparent, whereas in the control tube fluid was uniformly turbid (Pastushenko *et al*., 1977).

RESULTS

Sugar beet leaves, which were used for analysis, had prominent features of infection including necrotic, dark brown, nearly black spots with sizes ranging from several centimeters to the covering most of the leaf blade.

The bacteriological analysis resulted in isolation and identification of various morphological types of bacteria isolates. 41% of allocated isolates had colonies of yellow, 53% of gray and 6% of white color. 30% of isolates were shown to possess the ability to produce fluorescent pigment if they were cultivated on the King B medium.

A clear reaction of hypersensitivity on tobacco leaves was initiated by 7 isolates. A light brown zone was formed at the injection sites on the leaves at a 24-hour time point after suspensions of bacteria were introduced, which subsequently became darker. Leaf tissue in the zone of introduction of bacterial suspension became necrotic.

Bacteria that cause hypersensitivity reaction on tobacco leaves are phytopathogenic (Klement *et al*., 1990). Therefore, we consider the

isolates, which were isolated by us from sugar beet leaves with dark necrotic spots and caused hypersensitivity reaction, to be phytopathogenic. At the same time, it is necessary to determine their virulent properties.

The results of artificial inoculation of sugar beet plants in the phase of 2-3 pairs of leaves in the field conditions showed that the isolate «B-48-2» and other isolates identical to it (total of 7 isolates) cause the necrotic dark spot formation on the leaves, which gradually increases in size. In addition, the tissues inside of the spots get dry and fall out. All these isolates are Gram-negative and oxidase-negative motile rods that produce a fluorescent pigment. For a more detailed study of properties of identified isolates, we compared them with collection strains of bacterial pathogens of sugar beet.

The remaining studied isolates did not demonstrate pathogenic properties during artificial inoculation of sugar beet and bean plants.

In-depth analysis of the biological properties of pathogenic isolates was conducted in comparison with the collection strains and according to the data of Guide to bacterial determination (Bilai, 1988; Brenneu *et al*., 2005). It showed that by such criteria as Gram staining, motility, cell shape, presence of fluorescent pigment, absence of oxidase, aerobic use of glucose, absence of nitrate reduction and the formation of indole and hydrogen sulfide, the identified strains are identical to *Pseudomonas syringae.* The LOPAT scheme is commonly used for identification of *Pseudomonas syringae* and includes a number of features, in particular: L – levan production, O – oxidase production, P – pectinolitic activity, A – arginine dihydrolase production, T – development of reaction of hypersensitivity on tobacco leaves. Based on these features, we determined that the investigated pathogenic bacteria are identical to *Pseudomonas syringae*, as they produce levan, they are oxidase-

Table 1. Morphological, physiological and biochemical properties of bacteria

Tests	Isolates from sugar beet	*Pseudomonas syringae* [8]	*Pseudomonas syringae* pv. *aptata*
Gram staining	-	-	-
Motility	+	+	+
Cell shape	R	R	R
Sporulation	–	–	–
Fluorescent pigment	+	+	+
Oxidase	-	-	-
Pectinolitic activity	-	-	-
Reaction of hypersensitivity	+	+	+
Use of carbon sources: Glucose (anaerobic)	–	–	–
Glucose (aerobic)	+	+	+
Sucrose	–	×	–
Mannitol	–	×	–
Sorbitol	–	×	–
Inositol	–	×	–
Rhamnose	–	×	–
Arabinose	+	×	+
Arginine	–	×	–
Ornithine	–	–	–
Lysine	–	–	–
Arginine dihydrolase	–	–	–
Reduction of nitrates,	–	–	–
Indole production	–	–	–
H_2S production	–	–	–
Levan production	+	+	+

«+» - presence of property, «–» - absence of property, «X» – strains variability, «R» - rods.

negative, do not cause maceration of plant tissues, do not produce arginine dihydrolase and cause development of reaction of hypersensitivity on tobacco leaves. Regarding the spectrum of carbohydrate fermentation, this species is characterized by wide variability of strains in terms of sucrose, mannitol, sorbitol, inositol, rhamnose and arabinose (Table 1).

To confirm that allocated isolates belong to the species of *Pseudomonas syringae* we determined the fatty acid composition of total lipids of the bacterial cells, which is an important chemotaxonomic marker used for bacteria identification.

Table 2. Fatty acid composition of cells of isolates isolated from the infected leaves of sugar beet

Acid	Fatty acids content in the total area of peaks, %
3-hydroxydecanoic ($C_{10:0}$3OH)	less than 0,5%
dodecanoic ($C_{12:0}$)	3,31
2-hydroxydodecanoic ($C_{12:0}$ 2OH)	1,32
tetradecanoic ($C_{14:0}$)	0,66
hexadecenoic ($C_{16:1}$)	41,06
hexadecanoic ($C_{16:0}$),	36,43
octadecanoic ($C_{18:0}$)	0,66
octadecenoic ($C_{18:1}$)	16,56

Table 3. Results of agglutination reaction against strains from wheat and sugar beet with antiserums of various serogroups

Strain number	Titers of reaction with antiserums against strains *P. syringae*, serogroups (s.g)					
	UκM-1027s.g. I	κ-1025 s.g.II	Uκ B-1011 s.g. IV	8544 s.g. V	8545 s.g. IV	Uκ B-1115 s.g. VI
8544	n/d	n/d	n/d	1:25600	1:25600	n/d
8545	1:400	1:25600	1:1600	1:400	1:400	1:6400
B-48-2	1:400	1:400	1:12800	1:25600	1:25600	1:400
UκM - 1027	1:51200	n/d	n/d	–	–	–
-1025	n/d	1:25600	n/d	1:1600	1:800	n/d
Uκ B- 1011	n/d	n/d	1:6400	1:6400	1:6400	n/d
Uκ B-1115	n/d	n/d	n/d	1:800	1:6400	1:51200

«–» - agglutination reaction is absent, n/d- not determined.

The cells of isolates that were isolated from infected leaves of sugar beet were shown to contain fatty acids with the length of carbon chain from C_{10} to C_{18}. They include 3-hydroxydecanoic ($C_{10:0}$3OH), dodecanoic ($C_{12:0}$), 2-hydroxydodecanoic ($C_{12:0}$2OH), 3-hydroxydodecanoic ($C_{12:0}$3OH), tetradecanoic ($C_{14:0}$), hexadecanoic ($C_{16:0}$), hexadecenoic ($C_{16:1}$), octadecanoic ($C_{18:0}$) and octadecenoic ($C_{18:1}$) fatty acids, which quantitative and qualitative compositions are typical for *Pseudomonas syringae* (Table 2) (Stead, 1992).

DISCUSSION

Symptoms of infections that we observed on the sugar beet are similar to the symptoms described in the monograph (Gvozdiak *et al*., 2011) and are typical for the disease known as bacterial leaf spot of sugar beets.

Bacteria isolated from the infected leaves caused hypersensitivity reactions on tobacco leaves and their pathogenicity was confirmed on the host plant. Thus, we isolated bacteria that under natural conditions initiate pathological process in sugar beet.

Therefore, based on the morphological, cultural, biochemical and chemotaxonomic features the isolated bacteria are identified as members of the *Pseudomonas syringae* species, which initiate the infectious process once they are on the leaves of sugar beet, leading to appearance of dark necrotic spots. This has been experimentally confirmed by artificial infection of young plants.

We isolated *Pseudomonas syringae* strains and investigated them in agglutination reaction in test tubes with antisera against strains of four serogroups (I, II, IV, VI). Antiserum dilution against the strains of *P. syringae* pv. *atrofaciens* and *Pseudomonas syringae* pv. *aptata* in

homological reactions was quite high ranging from 1:25600 to 1:51200. The identified strains of *Pseudomonas syringae* showed an agglutination reaction with antisera of all serogroups in different titers, particularly, in the lowest titer of 1:400 with antisera against *P. syringae* pv. *atrofaciens* K-1025 (serogroup II), *P. syringae* pv. *atrofaciens* UKM B-1027 (I serogroup), *P. syringae* pv. *atrofaciens* UKM B-1115 (serogroup VI), and in the highest titer - 1:25600, with antisera against *Pseudomonas syringae* pv. *aptata* 8544, 8545 and 1:12800 with *P. syringae* pv. *atrofaciens* UKM B- 1011 (serogroup IV) (Table 3).

Thus, the strains isolated from sugar beet showed serological relationship with antiserum against strains-pathovars *P. syringae* of various serogroups. The high titer of the investigated strains in agglutination reaction with antiserums against representatives of serogroup IV indicates their affiliation to the serogroup IV according to the scheme of serogrouping of *P. syringae* bacteria (Pastushenko and Simonovych, 1979).

Researchers from different countries found that some aerobic, gram negative bacteria, including *Pseudomonas syringae* pathogens, which show epiphytic survival on apparently healthy plants, are characterized by a wide range of host plants and have an ice-forming ability (Hirano and Upper, 2000; Morris *et al*., 2007). The reason for this is the presence of specific membrane protein that allows the bacteria to form crystallization centers in supercooled water (Margaritis and Bassi, 1991). In France the strains of this species were isolated from snow, water for irrigation and various infected plants, including the non-agricultural plants. Therefore, there is an opinion that the spread of *Pseudomonas syringae* occurs with the rainfall or during the water cycle (Morris *et al*., 2007).

The number of infected plants with similar infection symptoms on the field was quite large. In this case, it can be assumed that the source of the pathogenic species *Pseudomonas syringae*, which we isolated from affected leaves of sugar beet, was the hail that had fallen the day before.

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