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ISOLATION OF ACTINOMYCETES WITH ANTAGONISTIC ACTIVITY AGAINST GRAM-NEGATIVE PHYTOPATHOGENIC BACTERIA

Research article

Abstract

Twenty eight actinomycetes cultures were isolated as separate colonies from phyto material of fruit trees with visual signs of bacterial infection and soil samples of Minsk region. The isolates were screened for antagonistic activity against plant pathogens from a specialized depository of phytopathogenic microorganisms Belarusian Collection of Non-Pathogenic Microorganisms (BIM). Two groups of actinomycetes cultures with antibacterial activity against phytopathogenic microorganisms of species *Pseudomonas corrugata* and *Pseudomonas syringae* were selected. In the short-term perspective, these strains may become the basis of "biorational" pesticides to control plant diseases.

Keywords: actinomycetes, antagonistic activity, plant pathogens, *Pseudomonas corrugata*, *Pseudomonas syringae*.

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ВЫДЕЛЕНИЕ АКТИНОМИЦЕТОВ С АНТИБАКТЕРИАЛЬНОЙ АКТИВНОСТЬЮ В ОТНОШЕНИИ ФИТОПАТОГЕННЫХ ГРАМОТРИЦАТЕЛЬНЫХ БАКТЕРИЙ

Научная статья

Аннотация

Из растительного материала плодовых деревьев с визуальными признаками бактериальной инфекции и образцов почвы Минской области до изолированных колоний выделены 28 культур актиномицетов. Проведен скрининг культур по признаку антагонистической активности в отношении 12 культур патогенов растений из специализированной коллекции фитопатогенных микроорганизмов фонда Белорусской коллекции непатогенных микроорганизмов (БКМ). Отобраны две группы культур актиномицетов, обладающих антибактериальной активностью в отношении фитопатогенных микроорганизмов видов *Pseudomonas corrugata* и *Pseudomonas syringae*. В перспективе, данные штаммы актиномицетов могут стать основой "биорациональных" пестицидов для защиты растений от возбудителей болезней.

Ключевые слова: актиномицеты, антагонистическая активность, патогены растений, *Pseudomonas corrugata*, *Pseudomonas syringae*.

1. Introduction

Biological control is application of microorganisms (or their metabolites) as natural antagonists of plant pests or pathogens to reduce or eliminate their harmful impact [1]. The use of biological control in agriculture is an effective strategy to combat plant pathogens damaging agro valuable crops. Fundamental studies on the mechanisms of molecular control of pathogenesis and biological control have revealed natural metabolites and chemical compounds triggering the development of "biorational" pesticides [2]. Actinomycetes represent a group of gram-positive filamentous bacteria [3], characterized by a complex cycle of morphological differentiation, accompanied by the production of numerous extracellular enzymes and various bioactive secondary metabolites [4], of high commercial significance in medicine and agriculture [5]. The group of actinomycetes is distinguished by the production of structurally diverse metabolites, like as β-lactam antibiotics, thienamycin, macrolides, streptomycin, erythromycin, anthracyclines, daunorubicin, doxorubicin, polyketides, rapamycin, tacrolimus, peptide

antibiotics, virginiamycin, pristinamycin, aminoglycosides, gentamicin and kanamycin [6]. Among different actinomycetes, genus Streptomyces attracts special attention of the researchers [7]. Amidst the existing spectrum of natural bio preparations possessing antibiotic activity, more than 10,000 compounds were isolated from actinomycetes. Of these, about 7600 were recovered from Streptomyces cells [8]. Streptomyces are known to be the sourcesv of many secondary metabolites with antibacterial, antifungal, antiparasitic, antitumor, and immunosuppressive properties [9]. Actinomycetes showing ubiquitous distribution [5] are especially widespread in soil and water. Isolation of actinomycetes from mixed soil microbiota is complicated by their slower growth as compared to other bacteria [10].

The aim of this study was isolation of new strains of actinomycetes and screening of displaying antagonistic activity against phytopathogenic gram-negative bacteria of genera *Pectobacterium, Pseudomonas, Xanthomonas, Serratia, Erwinia, Pantoea.*

2. Methods

2.1. Objects of study

Actinomycete strains isolated from soil and plant substrates served as the tested antibacterial culture. Bacterial strains *Pectobacterium carotovorum* BIM B-561, *Pseudomonas corrugata* BIM B-627, *Xanthomonas campestris* BIM B-634, *Pseudomonas syringae* BIM B-851, *Pseudomonas syringae* BIM B-855, *Xanthomonas sp.* BIM B-857, *Serratia sp.* BIM B-973, *Pseudomonas brassicacearum* BIM B-974, *Pseudomonas japonica* BIM B-976, *Pseudomonas reinekei* BIM B-977, *Erwinia sp.* BIM B-1087, *Pantoea sp.* BIM B-1094 from a specialized collection of phytopathogenic microorganisms Belarusian Collection of Non-Pathogenic Microorganisms acted as phytopathogenic agents.

2.2. Isolation and cultivation of strains

Plant materials were taken from fruit trees with visual signs of bacterial infection. Soil samples were collected from layers 10-15 cm deep in Minsk region. To isolate actinomycetes, 5 g of the sample was mixed with 50 ml of sterile distilled water and shaken for 30 min. A series of ten-fold dilutions was prepared from the resulting supernatant and 100 µl aliquots were plated on the selective agar medium with glycerol (Actinomycete Isolation Agar with Glycerol, Condalab), whereupon the plates were incubated for 7 days at 28 °C. The separate colonies of actinomycetes were distinguished morphologically, inoculated on GYM medium (yeast extract - 4 g; malt extract - 4 g; glycerol - 5 ml / l; agar - 15 g; pH 7.0 - 7.2) [11] and cultured for 5 - 7 days at 28 °C. Test cultures of phytopathogenic bacteria were incubated on meat-peptone agar for 24-48 h at 28 °C.

2.3. Cross Streak Method

Preliminary screening of actinomycetes for antibacterial activity in regard to phytopathogens was conducted using cross streak method. Actinomycete isolates shaped as a large central strip were seeded on Petri plates with Muller-Hinton Agar (MHA) and grown for 5-7 days at 28 °C. After 5-7 days phytopathogenic bacterial strains were inoculated across the central strip and the plates were re-incubated for 24-48 h at 28 °C. The antimicrobial activity of actinomycetes was evaluated via the presence of growth zones inhibition of phytopathogenic cultures around the central strip [12].

2.4. Agar Well Diffusion Method

A more detailed screening of actinomycetes for antibacterial activity toward phytopathogens was carried out using the wells technique. The analyzed actinomycete cultures were grown for 5-7 days at 28 °C in four liquid media: GYM, casein-starch medium (CSM): starch - 10 g; casein - 1 g; KNO_3 - 2 g; KH_2PO_4 - 2 g; NaCl - 2 g ; $MgSO_4$ x 7H2O - 0.5 g; $CaCO_3$ - 0.02 g; $FeSO_4$ x $7H_20$ - 0.001 g; agar - 18 g; pH 7.0 ~ 7.4), IM8 (glucose - 10 g; peptone - 5 g; tryptone - 3 g; NaCl - 5 g; agar - 15 g; pH 7.0) and a malt-yeast medium with $CaCO_3$ (glucose - 4 g; yeast extract - 4 g; malt extract - 10 g; $CaCO_3$ - 2 g; agar - 15 g; pH 7.0 ~ 7.4) [11]. Twenty-four hour cultures of phytopathogenic bacteria pre-grown in liquid meat-peptone medium at 28 °C were inoculated on Petri dishes with Müller-Hinton Agar (MHA) and actinomycete cultures after 5-7 days of incubation in liquid media were placed in to wells. The plates were incubated at 28 °C for 24-48 h. The antimicrobial activity of actinomycetes was estimated by the size of growth inhibition zones of phytopathogenic cultures formed around the wells [12].

3. Results

Twenty eight actinomycete cultures were isolated from samples according to morphological features, including seventeen bacteria from soil and eleven variants from the leaves of fruit trees with visual signs of bacterial infection (Table 1).

Table 1 – The list of actinomycete isolates and their isolation sources

Name of isolate culture	Source of isolation
Str1	pea field soil (Minsk region)
Str4	
Str5	
Str6	
Ger1	apple tree leaves (Minsk region)
Ap1	
Ap2	
Pe1	leaves of pear trees (Minsk region)
F2-1	apple tree leaves (Minsk region)
F2-2	
F2-3	
F3-1	leaves of plum trees (Minsk region)
F3-2	
F3-3	
F3-4	
LT1-1	sandy soil (Molodechno region)
LT1-2	
LT1-3	
LT1-4	
LT1-5	
LT1-6	
LT1-7	
LT1-10	
LT2-1	sod-podzolic soil (Molodechno region)
LT3-3	spruce rhizosphere (Molodechno region)
LT3-8	
LT4-1	silty soil (Molodechno region)
LT4-2	

Preliminary screening for antagonistic activity against plant pathogens has sorted out 12 actinomycete cultures: Str4, Ger1, Pe1, F2-2, F3-1, F3-2, LT1-2, LT1-3, LT1-4, LT1-10, LT2-1, LT4-1 (Table 2). These bacteria inhibited growth of phytopathogenic species Pseudomonas corrugata, Pseudomonas syringae and Xanthomonas sp., The size of growth inhibition zone ranged from 12 to 19 mm (Figure. 1).

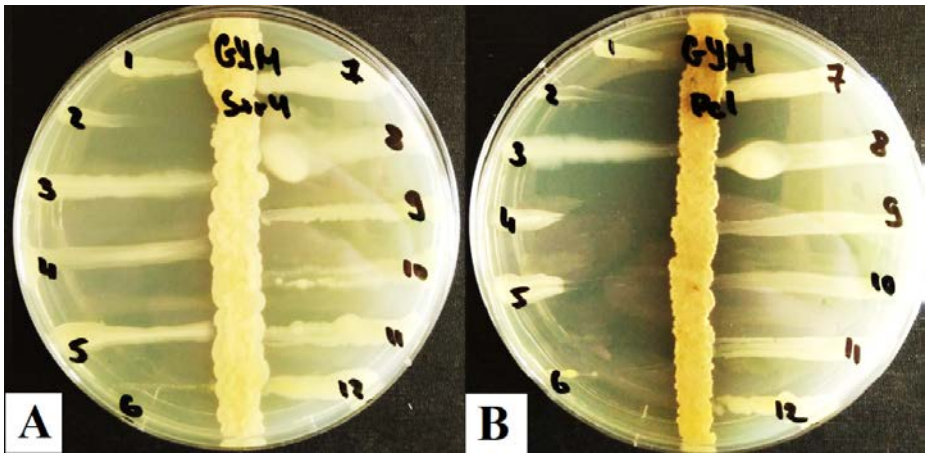

Figure 1 – Testing of isolates Str4 (*A*) and Pe1 (*B*) for resence of antagonistic activity against phythopatogenic bacteria by cross streak method

1. Pectobacterium carotovorum BIM B-561, 2. Pseudomonas corrugata BIM B-627, 3. Xanthomonas campestris BIM B-634, 4. Pseudomonas syringae BIM B-851, 5. Pseudomonas syringae BIM B-855, 6. Xanthomonas sp. BIM B-857, 7. Serratia sp. BIM B-973, 8. Pseudomonas brassicacearum BIM B-974, 9. Pseudomonas japonica BIM B-976, 10. Pseudomonas reinekei BIM B-977, 11. Erwinia sp. BIM B-1087, 12. Pantoea sp. BIM B-1094

Table 2 – Screening of isolates for resence of antagonistic activity against phythopatogenic bacteria by cross streak method *

Name of isolate	Presence of the inhibitory effect											
	Pectobacterium carotovorum B-561	*Pseudomonas corrugata* B-627	*Xanthomonas campestris* B-634	*Pseudomonas syringae* B-851	*Pseudomonas syringae* B-855	*Xanthomonas sp.* B-857	*Serratia sp.* B-973	*Pseudomonas brassicacearum* B-974	*Pseudomonas japonica* B-976	*Pseudomonas reinekei* B-977	*Erwinia sp.* B-1087	*Erwinia sp.* B-1094
Str1	–	–	–	–	–	–	–	–	–	–	–	–
Str4	–	+	–	+	+	–	–	–	–	–	–	–
Str5	–	–	–	–	–	–	–	–	–	–	–	–
Str6	–	–	–	+	+	–	–	–	–	–	–	–
Ger1	–	+	–	+	+	+	–	–	–	–	–	–
Ap1	–	–	–	–	–	–	–	–	–	–	–	–
Ap2	–	–	–	–	–	–	–	–	–	–	–	–
Pe1	–	+	–	+	+	–	–	–	–	–	–	–
F2-1	–	–	–	–	–	–	–	–	–	–	–	–
F2-2	–	+	–	+	+	+	–	–	–	–	–	–
F2-3	–	–	–	–	–	–	–	–	–	–	–	–
F3-1	–	+	–	+	+	–	–	–	–	–	–	–
F3-2	–	+	–	+	+	+	–	–	–	–	–	–
F3-3	–	–	–	+	+	–	–	–	–	–	–	–
F3-4	–	–	–	–	–	–	–	–	–	–	–	–
LT1-1	–	–	–	+	+	–	–	–	–	–	–	–
LT1-2	–	–	–	+	+	–	–	–	–	–	–	–
LT1-3	–	+	–	+	+	–	–	–	–	–	–	–
LT1-4	–	+	–	+	+	–	–	–	–	–	–	–
LT1-5	–	–	–	+	+	–	–	–	–	–	–	–
LT1-6	–	–	–	+	+	–	–	–	–	–	–	–
LT1-7	–	–	–	+	+	–	–	–	–	–	–	–
LT1-10	–	+	–	+	–		–	–	–	–	–	–
LT2-1	–	–	–	+	+	–	–	–	–	–	–	–
LT3-3	–	–	–	+	+	–	–	–	–	–	–	–
LT3-8	–	–	–	–	–	–	–	–	–	–	–	–
LT4-1	–	+	–	+	–	–	–	–	–	–	–	–
LT4-2	–	–	–	–	–	–	–	–	–	–	–	–

** Designations: '+' - presence of the inhibitory effect; '-' - lack of inhibitory effect*

Antagonistic activity of the selected actinomycetes cultures against phytopathogenic bacteria Pseudomonas corrugata BIM B-627, Pseudomonas syringae BIM B-851, Pseudomonas syringae BIM B-855, Xanthomonas sp. BIM B-857was studied using the agar well diffusion method (Table 3).

Table 3 – Screening of isolates for resence of antagonistic activity against plant patogens by agar well diffusion method

Name of isolate	Cultural medium	*Diameter of growth inhibition zone (mm)*			
		Pseudomonas corrugata B-627	*Pseudomonas syringae B-851*	*Pseudomonas syringae B-855*	*Xanthomonas sp. B-857*
Str4	GYM	14	4	–	–
	СД+$CaCO_3$	36	–	–	–
	CSM	22	–	–	–
	IM8	10	–	–	–
Pe1	GYM	12	4	–	–
	СД+$CaCO_3$	18	4	–	–
	CSM	18	–	–	–
	IM8	20	4	6	–
F2-2	GYM	10	4	4	6
	СД+$CaCO_3$	10	6	–	4
	CSM	12	8	8	–
	IM8	14	4	–	–
F3-1	GYM	10	8	10	6
	СД+$CaCO_3$	12	7	4	–
	CSM	18	6	4	–
	IM8	20	12	12	–
F3-2	GYM	16	8	6	–
	СД+$CaCO_3$	12	11	–	–
	CSM	22	6	6	–
	IM8	14	4	8	4
LT1-2	GYM	–	20	20	–
LT1-3	GYM	8	16	22	–
LT1-4	GYM	8	20	20	–
LT1-10	GYM	4	4	–	–
LT2-1	GYM	–	18	20	–
LT4-1	GYM	2	10	–	–
Ger1	GYM	10	6	4	10

The analysis revealed two groups of actinomycetes differing in inhibition targets. The first group showed a pronounced antagonistic effect against bacteria of the species Pseudomonas corrugata responsible for pathologies of tomato plants. Pathogenic bacteria infect plant core, causing necrosis - rotting of cell structure. The typical symptoms of infection are necrosis, swelling and softness of plant stem. On yeast peptone agar with glucose or nutrient dextrose agar, wrinkled colonies are formed with diffuse pigment yellow to brown in color [13]. The most pronounced antagonistic effect against bacterial pathogen was shown by actinomycete cultures Str4, Pe1, F3-1 and F3-2 (Figure 2).

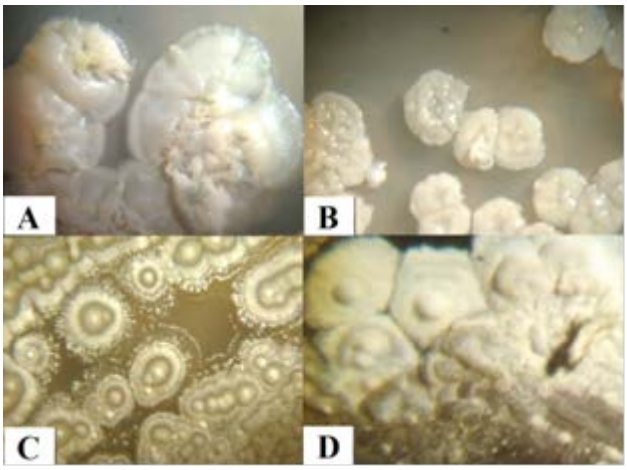

Figure 2 – Morphology of actinomycete colonies Str4 (*A*), Pe1 (*B*), F3-1 (*C*) and F3-2 (*D*)

During preliminary cultivation of actinomycete cultures on a yeast-malt medium with $CaCO_3$, CSM, and IM8 media, these isolates inhibited growth of Pseudomonas corrugata bacteria in the diameter ~ 18-32 mm (Figure 3).

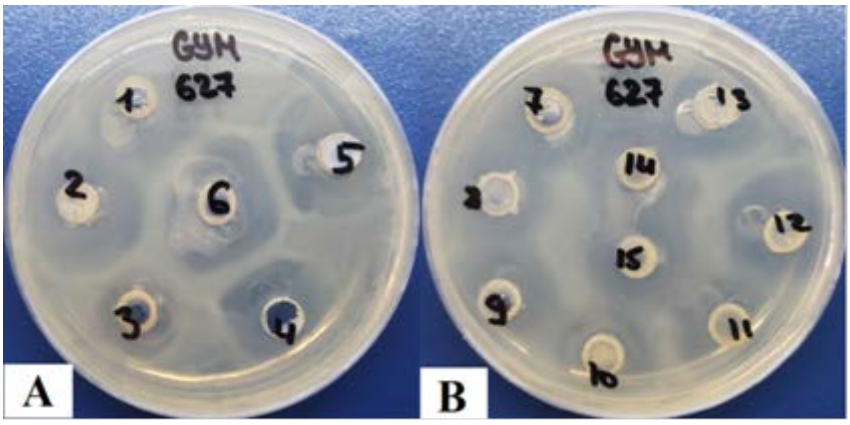

Figure 3 – Screening of actinomycetes for antagonistic activity against bacteria Pseudomonas corrugata BIM B-627 by the agar well diffusion method

A: 1. Str4 (СД+$CaCO_3$), 2. Str4 (CSM), 3. Str4 (IM8), 4.Str4 СД+$CaCO_3$), 5. Str4 (CSM), 6. Str4 (IM8);
B: 7. F2-2 СД+CaCO3), 8. F2-2 (CSM), 9. F2-2 (IM8), 10. F3-1 (СД+$CaCO_3$), 11. F3-1 (CSM), 12. F3-1 (IM8); 13. F3-2 (СД+$CaCO_3$), 14. F3-2 (CSM), 15. F3-2 (IM8);

The second group of tested cultures displayed a strong antimicrobial effect against bacteria of species Pseudomonas syringae. The latter are gram-negative plant pathogens, distinguished by diverse and host-specific interaction mechanisms. Over 60 pathovars identified so far are subdivided according to their ability to infect divers plant species. Pseudomonas syringae is one of the most common bacterial phytopathogens damaging phyllosphere. Microorganisms are capable to vegetate on the surface of plants in the form of epiphytic flora. In order to infect plants pathogens need to penetrate into apoplast through lesions or natural orifices, like stomata [14]. It was established that among tested actinomycetes isolates LT1-2, LT1-3, LT1-4, and LT2-1 displayed inhibitory effect on bacteria of species Pseudomonas syringae (Figure 4). These actinomycete cultures retarded growth of Pseudomonas syringae bacteria in the diameter of growth inhibition zone ~ 16-22 mm (Figure 5).

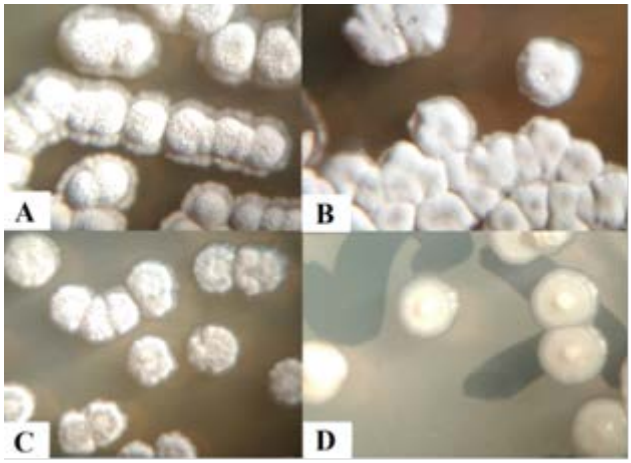

Figure 4 – Morphology of actinomycete colonies LT1-2 (*A*), LT1-3 (*B*), LT1-4 (*C*) and LT2-1 (*D*)

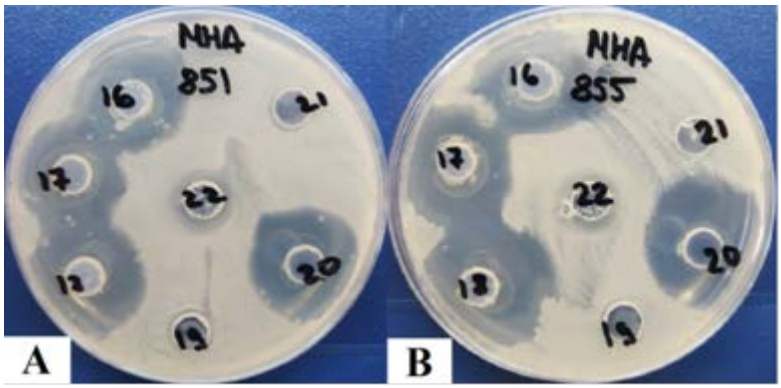

Figure 5 – Screening of actinomycete cultures for antagonistic activity Pseudomonas syringae B-851 (*A*), B-855 (*B*).
16. LT1-2, 17. LT1-3, 18. LT1-4, 19. LT1-10, 20. LT2-1, 21. LT4-1, 22. Ger1.

4. Conclusion

Screening for antibacterial activity against phytopathogenic strains Pectobacterium carotovorum BIM B-561, Pseudomonas corrugata BIM B-627, Xanthomonas campestris BIM B-634, Pseudomonas syringae BIM B-851, Pseudomonas syringae BIM B-855, Xanthomonas sp. BIM B-857, Serratia sp. BIM B-9731, Pseudomonas brassicacearum BIM B-974, Pseudomonas japonica BIM B-976, Pseudomonas reinekei BIM-977, Erwinia sp. BIM B-1087, Pantoea sp. BIM B-1094 preserved in a specialized depository of phytopathogenic cultures Belarusian collection of non-pathogenic microorganisms yielded 8 variants of actinomycetes demonstrating enhanced antagonistic action toward pathogenic species Pseudomonas corrugata (isolates Str4, Pe1, F3-1 and F3-2) and Pseudomonas syringae (isolates LT1-2, LT1-3, LT1-4 and LT2-1). The selected actinomycetes suppressed growth of bacteria in the diameter of growth inhibition zone equal to ~ 18-32 mm and ~ 16-22 mm, respectively, for each species. The promising actinomycete strains may be applied in elaboration of technologies for biological control of plant pathogens of species Pseudomonas corrugata and Pseudomonas syringae.

Conflict of Interest **Конфликт интересов**

None declared. Не указан.

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