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SCREENING OF SOIL STREPTOMYCETES – PRODUCERS OF ANTIBIOTICS AGAINST PHYTOPATHOGENIC BACTERIA

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Soil is an inexhaustible source of bacteria of the genus Streptomyces – the producers of the vast majority of known antibiotics that are successfully used in medicine, veterinary and agriculture. The emergence and spread of pathogenic bacteria resistance to antibiotics requires the search for new antibiotic compounds capable of overcoming this problem. ***Aim****. The purpose of this work was to isolate streptomycetes from soil samples of Kyiv and the Kyiv region and study their antibiotic activity against four strains of the different species of phytopathogenic bacteria.* ***Methods****. A suspension of soil in distilled water was sown on solid Chapek or corn-soybean medium in Petri dishes, in which trimethoprim and nystatin were introduced to inhibit bacterial and fungal growth. The antibiotic activity of the streptomycetes was tested by setting their agar discs on lawns of phytopathogenic bacteria in Petri dishes. Antibiotics were extracted from the streptomycetes agar cultures with a mixture of chloroform and acetone (2:1), dried in a rotary vacuum evaporator, dissolved in ethanol, separated and purified by thin layer chromatography on aluminum plates (Silica gel 60* F_{254} *from Merck KGaA). The UV/Vis absorption spectra of the antibiotics were measured with a Beckman DU 8 spectrophotometer.* ***Results.*** *10 strains of streptomycetes were isolated from the soil samples of Kyiv and the Kyiv region, whose antibiotic activity was tested against four phytopathogenic bacteria using the agar block method. Three of the streptomycetes – B8, SK and KZ, formed growth inhibition zones of different phytopathogens on complete medium in Petri dishes, among which the strain SK was the most active. This strain showed antibiotic activity against all four phytopathogenic bacteria – P. syringae 8511, P. carotovorum 8982, C. michiganensis 10 and X. campestris 8003.* ***Conclusions****. The results obtained are of interest for the protection of sensitive plants by isolated antibiotics against phytopathogenic bacteria in hothouse conditions.*

Keywords: soil streptomycetes, antibiotic activity, phytopathogenic bacteria, thin layer chromatography, absorption spectra of antibiotics.

Search for new antibiotics remains an actual and important medical and biological problem due to the appearance of the antibiotic-resistant microorganisms [1]. The reason for the emergence of antibiotic-resistant microorganisms is the excessive or incorrect use of antibiotics as medicines. Many bacteria belonging to the *Streptomyces* genus are industrially important producers of antibiotics used in medicine, veterinary and agriculture [2, 3]. Streptomycetes make up to 40 % of soil microorganisms and only a very small part of them have been investigated [4].

The aim of this work was to isolate the *Streptomyces* strains from soil samples of Kyiv and the Kyiv region to investigate the properties of their antibiotics, specificity of their activity against phytopathogenic bacteria.

Materials and methods. Samples of surface soil at a depth of up to 2 cm were collected in sterile tubes in the vicinity of Kyiv. One gram of soil sample was diluted in proportions of 1:10 and 1:100 in sterile distilled water and the resulting suspensions were plated on Chapek medium or corn-soy medium (g/l: corn meal – 20.0, soy meal – 10.0, NaCl – 5.0, agar – 15.0, distilled water – 1.0 l, sterilization at 121° C for 30.0 min) in Petri dishes in which trimethoprim and nystatin were introduced to inhibit bacterial and fungal growth. The dishes were incubated in a thermostat at 28° C for one week and isolated colonies of Streptomycetes were sown on slant corn-soy agar in the test tubes for use in the next investigation.

Preliminary antibiotic activity of the isolated streptomycetes was determined using agar discs from lawns of 5–day cultures, laid on the surface

of agar containing test cultures of phytopathogenic bacteria – *Pseudomonas syringae* 8511*, Pectobacterium carotovorum* 8982*, Clavibacter michiganensis* 10 and *Xanthomonas campestris* 8003, obtained from Ukrainian Collection of Microorganisms (UMU) [5]. After the selection of the most active strains, the antibiotic activity of the agar extracts was investigated. Lawns of 5-day culture were cut into 1.0x1.0 cm cubes and antibiotic compounds were extracted with chloroform-acetone (2:1), dried in a rotary vacuum evaporator and dissolved in ethanol. The antibiotic activity of the obtained crude extracts was checked by cutting holes in an agar medium S (g/l: peptone – 4.0, yeast extract – 4.0, K_2HPO_4 – 0.5, $MgSO_4$ – 0.25, agar – 15.0, distilled water – 1.0 l) in Petri dishes with test cultures. 20 µl of crude extract in ethanol was added in each hole. The number of individual components in the unknown antibiotics of each raw material was determined by thin layer chromatography (TLC), using 20x20 cm aluminum plates from Merck KGaA (Silica gel 60 F_{254}) and a solvent system of butanol-ethyl acetate-acetone-ethanol (4:2:1:0.5). Separate stripes of antibiotics, clearly visible in UV light, were removed with silica gel from the plates, dissolved in dilute ethanol (1:1) or spread onto the surface of the agar containing individual cultures of phytopathogenic bacteria. The ethanol solutions of the purified antibiotics were used to obtain absorption spectra by means of the Beckman DU 8 spectrophotometer.

Results. 10 strains of streptomycetes were isolated from soil samples of Kyiv (Feofaniya) and the Kiev region (Brovary). The most active strains B8, SK and KZ were isolated for further study after initial testing for antibiotic activity. Fig. 1 shows the growth inhibition zones of three phytopathogenic bacteria around blocks of agar cultures of five strains of streptomycetes.

F i g. 1. Areas of inhibition of growth of *P. syringae* 8511 (1), *X. campestris* 8003 (2) and *C. michiganensis* 10 (3) around blocks of agar cultures of strains of streptomycetes: K15 (1), KZ (2), B8 (3), 277 (4), K70 (5)

As can be seen from this figure, only strain B8 showed antagonistic activity against all three phytopathogenic bacteria – *P. syringae* 8511*, X. campestris* 8003 and *C. michiganensis* 10. Strains KZ and 277 were active antagonists only against individual phytopathogenic bacteria. In another experiment, the highest antagonistic activity against all four phytopathogenic bacteria was found in SK streptomycete strain (Table 1).

Antibiotic compounds of all three streptomycete strains – B8, KZ and SK were extracted with a mixture of chloroform and acetone (2:1) from 5–day agar cultures, dried in a rotary evaporator and dissolved in 1:1 diluted ethanol. Fig. 2 presents the growth inhibition zones of *P. syringae* 8511 around the hole in the agar, containing 80 µl of the extract of agar culture B8.

Unknown antibiotic compounds were detected and separated by thin layer chromatography on Merck aluminum plates (Silica gel 60 F_{254}) in the solvent system butanol : ethyl acetate : acetone : ethanol (4:2:1:0.5). Fig. 3 presents a chromatographic plate depicting the individual strips of compounds from the culture extract of the strains SK and KZ visible in UV light.

Strips 1 (blue), 2 (dark) and 3 (blue) on plate A were collected separately with gel powder, then dissolved in dilute ethanol, and 80 µl of the each solution was placed in the holes on the *C. michiganensis* 10 culture lawn (Fig. 4).

Table 1

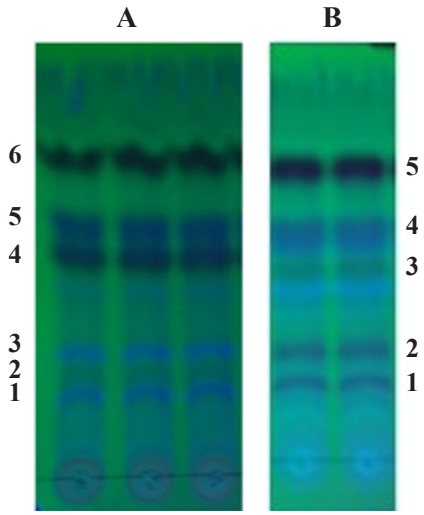
Some properties of streptomycete antibiotics

Strain	Soil sample	*P.syringae*	*P. carotovorum*	*C.michiganensis*	*X.campestris*	λ max, nm
B8	Kyiv, Feofania	20,0*	16,0	15,0	24,0	264,0
K15	Kyiv region	0,0	15,0	28,0	0,0	258,0
SH24	« «	0,0	15,0	22,0	0,0	-
B35	« «	0,0	20,0	27,0	16,0	-
SK	Brovary	28,0	30,0	35,0	28,0	242,0
K70	Bobrytsia	0,0	20,0	0,0	0,0	258,0
Z175	«	0,0	18,0	16,0	14,0	-
KZ	«	0,0	22,0	30,0	0,0	262,0
37	Kyiv region	0,0	18,0	17,0	15,0	-
277	Reshetylivka	18,0	18,0	0,0	0,0	-

* Zone diameter (mm) of no growth around the block of Streptomyces agar culture

F i g. 2. Zone of absence of growth of *P. syringae* 8511 around the holes with the culture extract of streptomycete strain B8 (1) and the growth of bacteria around the holes with extracts of cultures of strains B35 (2), K70 (3) and KZ (4)

F i g. 4. Areas of absence of growth of *C. michiganensis* 10 around the holes with antibiotics of streptomycete strain SK collected from stripes 1, 2 and 3 of TLC (Fig. 3, A)

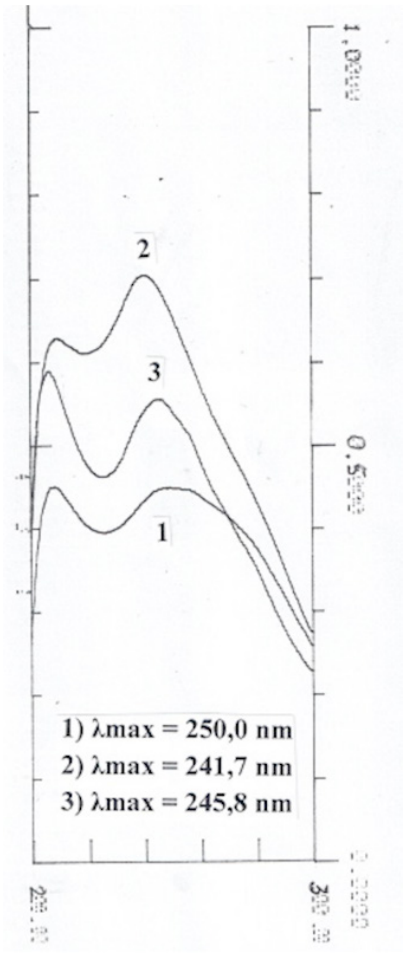


F i g. 3. Thin layer chromatograms of extracts of streptomycete cultures SK (A) and KZ (B) in UV light

As can be seen in this figure, all three compounds exhibited antibiotic activity, which was highest in the upper compound. The agar culture extract of KZ strain produces 5 stripes visible in UV light (Fig. 3, B) on the TLC plate. Antibiotic activity against *P. carotovorum* 8982 was demonstrated by the compounds in band 2 (Rf 0.27) and 4 (Rf 0.70). Compounds in stripes 2 and 5 inhibited the growth of *C. michiganensis* 10.

The absorption spectra of compounds 1, 2 and 3 of the agar culture extract of SK streptomycete strain are presented in Fig. 5. They have absorption maxima 250.0 nm, 245.8 nm and 242.0 nm, respectively.

KZ streptomycete strain forms an antibiotic-active compound whose delay factor (Rf) and absorption maximum (λ) are 0.70 and 262.0 nm, respectively.



F i g. 5. Absorption spectra of antibiotics of streptomycete strain SK collected from stripes 1, 2 and 3 of TLC (Fig. 3, A)

Discussion. Soil exhibits an inexhaustible abundance of microorganisms, among which streptomycetes occupy one of the leading places in number and value [2]. Today, only 1 % of these bacteria, important for soil fertility and new antibiotic producers finding, have been investigated by scientists [4]. Due to the spread of antibiotic-resistant pathogens [1], streptomycetes will still be used for a long time by scientists to find new antibiotic compounds for use in medicine, veterinary and agriculture [3].

10 strains of streptomycetes with antibiotic activity on up to four phytopathogenic bacteria were isolated from the soil samples taken in Kyiv and the Kyiv region. The most active antagonists of phytopathogenic bacteria were found to be the strains of streptomycetes – B8, SK and KZ (Table 1). The most sensitive to the antibiotics of these three strains of streptomycetes was gram-positive bacterium *C. michiganensis* 10, while gram-negative bacteria *P. syringae* 8511, *P. carotovorum* 8982 and *X. campestris* 8003 formed smaller zones of growth absence under the influence of the above antibiotics. Among the gram-negative bacteria *P. syringae* 8511 proved to be the most resistant to the isolated antibiotics.

By means of thin layer chromatography of the streptomycetes agar extracts separate purified antibiotic fractions were obtained. Their Rf retention factors and absorption spectra in UV light were determined (Table). The absorption maxima of the antibiotics of strains B8, SK and KZ are 264.0, 242.0, and 262 nm, respectively, and the Rf values are 0.52, 0.38, and 0.70, respectively.

Based on the data obtained, the following scientific studies can be planned in the future: 1) selection of more active antibiotic producers by the induced mutagenesis of the strains of streptomycetes B8, SK and KZ; 2) selection of optimal conditions for streptomycetes strains growing (medium composition and intensity of aeration) in shaking flasks; 3) selection of optimal conditions for the extraction of antibiotics from culture fluid; 4) checking the protective effect of antibiotics against phytopathogenic bacteria-induced infection of susceptible plants in hothouse conditions; 5) study of the ability of isolated antibiotics to overcome the resistance of phytopathogenic bacteria to known antibiotics.

Conclusions. 10 strains of streptomycetes were isolated from soil samples of Kyiv and the Kyiv region and their antibiotic activity against four phytopathogenic bacteria was investigated. Blocks of agar cultures of three selected strains of streptomycetes – B8, SK and KZ formed the largest growth inhibition zones of phytopathogenic bacteria. Strain SK showed the most active bactericidal action on all four strains of phytopathogenic bacteria – *P. syringae* 8511, *P. carotovorum* 8982, *C. michiganensis* 10 and *X. campestris* 8003. B8 strain was less active in this respect, and KZ strain effectively suppressed the growth of only *C. michiganensis* 10 and, to a lesser extent, *P. carotovorum* 8982. Using thin layer chromatography, the antibiotic compounds of agar culture extracts of B8, SK and KZ strains were separated and purified on Merck KGaA aluminum plates Silica gel 60 $F_{254,}$ and their UV/Vis absorption spectra were determined using a Beckman DU 8 spectrophotometer. Testing the ability of the isolated antibiotics to protect sensitive plants against phytopathogenic bacteria in hothouse conditions is of interest, as well as establishing their chemical structure.

ПОШУК ҐРУНТОВИХ СТРЕПТОМІЦЕТІВ – ПРОДУЦЕНТІВ АНТИБІОТИКІВ ДО ФІТОПАТОГЕННИХ БАКТЕРІЙ

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Резюме

Ґрунт є невичерпним джерелом бактерій роду *Streptomyces* – продуцентів значної більшості відомих антибіотиків, які успішно застосовуються в медицині, ветеринарії і сільському господарстві. Виникнення і поширення резистентності патогенних бактерій до антибіотиків вимагає пошуку нових антибіотичних сполук, здатних подолати дану перешкоду. **Мета**. Метою даної роботи було виділення зі зразків ґрунту Києва і Київської області стрептоміцетів і дослідження їх антибіотичної активності по відношенню до чотирьох штамів різних видів фітопатогенних бактерій. **Методи**. Висів суспензії ґрунту в дистильованій воді на тверде кукурудзяно-соєве середовище в чашках Петрі, яке містило триметопрім і ністатин з метою пригнічення росту бактерій і грибів. Антибіотична активність стрептоміцетів перевірялася шляхом накладання дисків агарових культур останніх на газони фітопатогенних бактерій в чашках Петрі. Антибіотики екстрагували з агарових культур стрептоміцетів сумішшю хлороформу і ацетону (2:1), висушували в роторному вакуумному випарникові, розчиняли в етанолі, розділяли і очищували за допомогою тонкошарової хроматографії на алюмінієвих пластинках Silica gel 60 F_{254} фірми Merck. Спектри поглинання антибіотиків в УФ світлі визначали за допомогою спектрофотометра Beckman DU 8. **Результати**. Зі зразків ґрунту Києва і Київської області виділено 10 штамів стрептоміцетів, антибіотичну активність яких проти чотирьох штамів фітопатогенних бактерій перевірено за допомогою методу агарових блоків. Три штами стрептоміцетів – В8, СК і КЗ утворювали зони відсутності росту різних фітопатогенів на повноцінному середовищі в чашках Петрі, серед яких найбільш активним був штам СК. Даний штам проявляв антибіотичну активність по відношенню до усіх чотирьох штамів фітопатогенних бактерій – *P. syringae* 8511*, P. carotovorum* 8982*, C. michiganensis* 10 і *X. campestris* 8003. **Висновки**. Одержані результати представляють інтерес для дослідження захисту чутливих рослин виділеними антибіотиками від фітопатогенних бактерій в умовах теплиці.

Ключові слова: ґрунтові стрептоміцети, антибіотична активність, фітопатогенні бактерії, тонкошарова хроматографія, спектри поглинання антибіотиків.

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