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STEROLS BIOSYNTHESIS BY SOIL STREPTOMYCETES

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The goal of this work was to study biosynthesis of sterols by soil streptomycetes Streptomyces avermitilis IMV Ac-5015, Streptomyces netropsis IMV Ac-5025 and Streptomyces violaceus IMV Ac-5027 on various media under submerged cultivation and to determine their content in bioformulations, developed on the basis of these metabolites producers. Analysis of sterol derivatives extracted from biomass, supernatant of cultural liquid and biological products were performed by GC/MS. The biomass of streptomycetes contained sterols in significantly higher amounts than in the cultural supernatants, and their spectrum and quantitative ratio were different. Squalene – sterol precursor – was found in the biomass in quantities producers 6.2-43.3 μg/g, when streptomycetes grown in synthetic and 8.2-212.1 μg/g in organic media. The biomass of S. avermitilis IMV Ac-5015 was distinguished by domination of 24-epibrassinolide, the content of which was the highest among the tested strains and reached 268.2 μg/g in organic and 345 μg/g in synthetic media. S. avermitilis IMV Ac-5015 did not synthesize sitosterol and stigmasterol that is important in respect of nematicidal properties of the strain. The biomass of S. netropsis IMV Ac-5025 and S. violaceus IMV Ac-5027 grown on organic medium contained 2.3-6.8-fold higher amount of sitosterol and 1.5-3-fold higher amount of stigmasterol compared to synthetic medium. Ergosterol prevailed in sterol spectrum of S. netropsis IMV Ac-5025 and S. violaceus IMV Ac-5027. The highest total content of sterols (9.5 mg/l) was found in Avercom (producer – S. avermitilis IMV Ac-5015). The use of exogenous sterols of microbial origin is important for regulation of their ratio in plants and for increasing resistance to pathogens and phytonematodes.

K e y w o r d s: soil streptomycetes, sterols, inducers of plant resistance, phytopathogens, phytonematodes.

Steroid compounds play an important role in vital activity of warm-blooded animals, plants and microorganisms. Squalene is a precursor of sterols, it turns into cyclic lanosterol ($C_{30}H_{50}O$) or its isomer cycloartenol, various sterols which contain 27-29 carbon atoms being formed of them [1]. Sterols are structural elements of biologic membranes, besides, they perform important regulatory functions. Cholesterol is the main sterol in animals; steroid hormones, bile acids, lipoproteins and vitamin D are formed of it. The content of sterols is more diverse in vascular plants [2, 3]. There are β-sitosterol, stigmasterol and campesterol, which are the main ones, cholesterol and ergosterol may be present as well [3, 4]. It is known that sterols are precursors of plant hormones – brassinosteroids, which extra-low concentrations (10^{-9}–10^{-12} M) affect the broad spectrum of plant cell responses [5]. Brassinosteroids raise plant adaptation abilities: stimulate expression of protective genes, resistance to diseases, to unfavorable factors of the environment, including that to negative effect of pesticides [6-8]. Microorganisms' capacity to synthesize sterols still remains poorly studied. It is known from literature that ergosterol is the main sterol in the membranes of yeast and micelium fungi; it plays the same role as cholesterol in the cells of animals [9]. Sterols of the group of hopanoides, affecting permeability and plasticity of membranes and other properties like sterols of eukaryotes have been found in bacteria [10-12]. In most bacteria hopanoide can take part in correction of permeability of cell membranes, in adaptation to extreme conditions of the environment. They are formed in the air hyphas and spores of prokaryotic soil bacteria of *Streptomyces* genus, where they help minimize the loss of water through the membrane into the air.

Mycelial soil actinobacteria, widely distributed in nature, are a source of biologically active substances, which are different as to chemical structure and spectrum of their effect. They include representatives of *Streptomyces* genus synthesize substances taking antibacterial, antifungal and antiparasitic effect, as well as other biologically active substances as amino acids, enzymes, vitamins, lipids, phytohormones [14, 15]. But steroid compounds were almost not detected among the broad spectrum of biologically active substances synthesized by streptomycetes.

Thus, the work objective was to study biosynthesis of steroid compounds by soil streptomycetes *S. avermitilis* IMV Ac-5015, *S. netropsis* IMV Ac-5025 and *S. violaceus* IMV Ac-5027 under different cultivation conditions at deep growing and to determine their content in biological preparations developed on the basis of metabolites of the strains under study.

Materials and Methods

Streptomycetes *S. avermitilis* IMV Ac-5015, *S. netropsis* IMV Ac-5025, S. violaceus IMV Ac-5027 isolated from the chernozem and southern chestnut soils are the objects of research. In our previous works [15, 16] we presented data concerning screening, identification and selection of these strains, which are characterized by high antagonistic activity against of phytopathogenic fungi (*Alternaria alternata* and *Fuzarium oxysporum*), phytopathogenic bacteria of genera *Pseudomonas, Xanthomonas*, *Pantoea*, *Clavibacter* and phytonematodes of genera *Meloidogyne*, *Ditylenchus*, *Pratylenchus*, *Tylenchobrynchus*, *Helicotylenchus*, *Paratylenchus*, *Heterodera*. Besides antagonistic features the strains also demonstrate phytostimulating and adaptogenic properties that is promising for creation on their basis of environmentally friendly metabolic biological preparations for plant growing [15, 17, 18]. New polyfunctional metabolite preparations Avercom (producer – *S. avermitilis* IMV Ac-5015), Phytovit (producer – *S. netropsis* IMV Ac-5025) and Violar (producer – *S. violaceus* IMV Ac-5027) have been developed on the basis of the complex of biologically active substances. The preparations combine antagonistic activity against phytopathogens and phytonematodes, as well as the properties of plant growth regulators and adaptogens [17, 18].

To investigate biosynthesis of sterols streptomycetes were grown in liquid synthetic (starch-ammonia) and organic (soya) media [15] during 7 days. The strains in exponential phase of growth, grown in soya medium, were used for inoculation. The amount of seeding material was 5% of the volume of liquid nutrient medium. Cultivation was performed in Erlenmeyer flasks of 750 ml with 100 ml of the medium on rotor rockers (28 ± 1 °C; n = 240 rev/min) to stationary growth phase. Biomass of streptomycetes was separated by centrifugation (4000 g) during 20 min. The cultural liquid supernatant was kept at 4 °C. To obtain ethanol extract 10 ml of distilled water cooled to 4 °C were thoroughly mixed and centrifuged for 10 min at 4000 g. The biomass was washed off 3-4 times. Ethanol (96%) in the ratio of 1 : 5 was added to washed-off biomass, and under periodic mixing extraction was executed at room temperature during 24 h, after that the biomass was separated by centrifugation (10 min, 4000 g). The obtained ethanol extract was stored at 4 °C [15]. Supernatants of cultural liquid and ethanol extracts of biomass of streptomycetes were used for determining the content of sterols and for creation of the corresponding compositional metabolic biological preparations.

The preparation Violar included supernatant of cultural liquid and ethanol extract of the biomass (4:1) of *S. violaceus* IMV Ac-5027. The preparation Phytovit is composed by ethanol extract of the biomass and supernatant of cultural liquid of *S. netropsis* IMV Ac-5025. Avercom is ethanol extract from the biomass of *S. avermitilis* IMV Ac-5015. Our previous investigations have detected antibiotic substances of different chemical nature in the composition of developed metabolic biological preparations: macrolide avermectins in Avercom, anthracycline ones in Violar, polyene ones (tetraene and pentaene) in Phytovit. Besides, all the elaborated biological preparations include biologically active substances, synthesized by producers: amino acids, lipids, including phospholipids, sterines, fatty acids, as well as phytohormones (auxins, cytokinins, gibberelins), etc. [17, 18].

Determination of compounds of steroid nature. Cultural liquid medium was centrifuged at 0 °C and 10 000 g during 20 min. The extraction by ethyl alcohol from producer biomass was conducted as described above [15]. The ethanol extract was concentrated by evaporation in vacuum at 45 °C. Sterols from dry residue were thrice extracted by mixture acetonitrile/ethylacetate (1 : 1 rev/rev). Extracts were concentrated with evaporation in

vacuum, then the samples were prepared following the protocol [19]. Preliminarily prepared and dried samples were dissolved in 100 µl of pyridine adding 100 µl of the reaction mixture – N,O-bis (trimethylsilyl) trifluoroacetamide/trimethylchlorosilane (5/1, v/v), held during 30 min at 70 °C (derived) [20]. Steroid compounds were analyzed by the method of gas chromato-mass spectroscopy using the device 6890N/5973inert (Agilent Technologies, USA). Chromatographic separation was carried out on the capillary column HP-5ms (30 m×0.25 mm×0.25 µm, J&W Scientific, USA) in gradient conditions. Initial temperature 275 °C was maintained during 16 min, with next gradient – 20 °C/min to 300 °C, with plateau 5 min. Gas carrier was helium, flow rate through the column was 1 ml/min, a sample of 1 µl was introduced in conditions of flow division with coefficient 1 : 50. The temperature of evaporator was 250 °C, of interface – 280 °C. Ionization was carried out in conditions of electron shock with energy of 70 eV, recording of ions was performed in SCAN conditions within the range of 50-600 m/z. The data were fixed and processed using software ChemStation, the components under study were identified with the help of mass-spectrum library NIST 02 and corresponding standards of cholesterol, ergosterol, sitosterol, stigmasterol and 24-epibrassinolide (Sigma-Aldrich).

Calculations and statistical processing of obtained data were made using computer programs Statistica 6.0 and Microsoft Excel '10.

Results and Discussion

Sterols, which spectrum and quantitative ratio were different in the strain, were found in biomass of all the studied producers and in supernatant of cultural liquid (Table 1).

Squalene was found in the supernatant of cultural liquid in inconsiderable amount (from 0.83 to 2.11 µg/g). Its content was considerably higher in biomass of producers and was 16.18-43.32 µg/g, when streptomycetes were grown in synthetic medium and 8.17-212.13 µg/g under growth in organic medium.

Qualitative composition of steroidogenesis end products evidences that the strains studied synthesized sterols in different independent ways. In particular, probably, squalene is oxidized to 2.3-oxidos-

Table 1. Biosynthesis of sterols by soil streptomycetes

Steroid compounds	*S. avermitilis* IMV Ac-5015		*S. netropsis* IMV Ac-5025		*S. violaceus* IMV Ac-5027	
	Content of sterols, µg in ml of medium or in g ADB***					
	1*	2**	1	2	1	2
Synthetic growing medium						
Squalene	nd	nd	2.11 ± 0.11	43.32 ± 2.19	0.83 ± 0.04	16.18 ± 1.34
Cholesterol	0.33 ± 0.02	106.85 ± 3.44	0.45 ± 0.02	16.75 ± 1.36	0.48 ± 0.02	9.41 ± 1.02
Ergosterol	0.04 ± 0.01	11.98 ± 1.15	0.88 ± 0.04	60.25 ± 2.59	0.96 ± 0.05	65.79 ± 2.70
Sitosterol	nf	nf	0.16 ± 0.01	29.71 ± 1.82	0.18 ± 0.01	10.74 ± 1.09
Stigmasterol	nf	nf	nf	0.68 ± 0.03	nf	3.58 ± 0.63
24-epibrassinolide	nf	345.03 ± 6.19	nf	4.13 ± 0.68	nf	4.84 ± 0.3
Organic growing medium						
Squalene	nd	nd	1.58 ± 0.08	212.13 ± 4.85	1.08 ± 0.05	8.17 ± 0.89
Cholesterol	1.17 ± 0.06	192.60 ± 4.63	0.57 ± 0.03	131.45 ± 3.82	0.56 ± 0.03	4.94 ± 0.74
Ersterol	0.19 ± 0.01	93.44 ± 3.22	1.66 ± 0.08	398.86 ± 6.66	0.66 ± 0.03	159.23 ± 4.21
Sitosterol	nf	nf	1.73 ± 0.09	201.21 ± 4.73	1.65 ± 0.08	25.13 ± 1.67
Stigmasterol	nf	nf	0.38 ± 0.02	2.02 ± 0.47	0.35 ± 0.02	5.24 ± 0.76
24-epibrassinolide	10.74 ± 0.54	268.16 ± 5.46	0.25 ± 0.01	8.16 ± 0.95	0.08 ± 0.01	1.49 ± 0.41

Note:"nd" – not determined, "nf" – not found, *1 – supernatant of cultural liquid (µg/ml), **2 – biomass (µg/g), ***ADB – absolutely dry biomass

qualene with further formation of cyclic lanosterol, the following products being synthesized of it in three independent ways: a) sitosterol and stigmasterol (through isofucosterol), b) brassinisteroid 24-epibrassinolide (through campestrol) and c) ergosterol. Cholesterol availability among metabolites evidences that the studied streptomycetes were able to form from 2.3-oxidosqualene – cycloartenol, the end product cholesterol being synthesized from the latter.

Sterols were found in supernatant of cultural liquid in considerably lower amounts compared with biomass. It was shown that sterols were mainly accumulated in producers' biomass and were not practically produced into the growing medium. Cholesterol and ergosterol content in biomass of *S. avermitilis* IMV Ac-5015, grown in synthetic medium, was 1.8 and 7.8 times lower than in organic one, while the content of 24-epibrassinolide was, on the contrary, 1.3 times higher. Among sterols contained in biomass of *S. avermitilis* IMV Ac-5015 one could observe predomination of 24-epibrassinolide, which content reached 268.16 μg/g (in organic) and 345.03 μg/g (in synthetic medium) and was the highest among the studied strains. As soon as synthesis of brassinosteroids, on the one hand, and sitosterol and stigmasterol, on the other hand, proceed in parallel ways from a common precursor lanosterol, the found high synthesis of 24-epibrassinilide by *S. avermitilis* IMV Ac-5015 can evidence for blocking the parallel way.

The fact that *S. avermitilis* IMV Ac-5015 does not synthesize sitosterol, which is a precursor of stigmasterol synthesis, is important from the viewpoint of practical use of the strain. It is known that stigmasterol is a dominating sterol in eggs and females of phytoparasitic nematodes. Helminths cannot produce this sterol, but consume it from plants during invasion. This determines the possibility of existence and propagation of parasites and extent of realization of their species biologic potential [6-8]. Thus, the absence of sitosterol and stigmasterol retains propagation of nematodes. Sitosterol and stigmasterol were found both in the biomass and supernatant of cultural of *S. netropsis* IMV Ac-5025 and *S. violaceus* IMV Ac-5027, grown in synthetic and organic media. The content of sitosterol and stigmasterol was 6.8 and 3.0 times higher in the biomass of *S. netropsis* IMV Ac-5025 grown in organic medium compared with synthetic one. The same tendency was characteristic of *S. violaceus* IMV Ac-5027 – synthesis of sitosterol and stigmasterol in organic medium was 2.3 and 1.5 times higher than in synthetic medium.

Ergosterol prevailed in sterols found in the biomass of strains *S. netropsis* IMV Ac-5025 and *S. violaceus* IMV Ac-5027. It is known that ergosterol is always exceptionally detected in the content of cell membranes of fungi, where it, in addition, is a dominant sterol. Like other sterols it regulates the conformation changes of membranes and strains the survival of microorganisms in unfavorable conditions. It should be emphasized that phytopathogenic oomycetes (*Phytophthora* and *Pythium*) cannot synthesize sterols and their membranes incorporate sterols taken from the host-plant. It means that they depend on exogenic sterols, but the extent of their dependence and correlation between the level of plant resistance and a certain content of sterols is open to question [7, 8]. The data concerning synthesis of sterols by the studied streptomycetes have been obtained for the first time.

The total amount of sitosterol and stigmasterol, synthesized by the strains *S. netropsis* IMV Ac-5025 and *S. violaceus* IMV Ac-5027 was higher as compared with 24-epibrassinolide that points to competitive inhibition of the parallel way of synthesis of the latter from cyclic lanosterol.

Hence the supernatant of cultural liquid and biomass of strains selected by the authors contain the considerable quantity of steroid compounds, being of great practical importance, and may be used in plant growing.

It is currently noted that more and more researchers support the thought that isoprenoid compounds not only appear as the regulators of plant growth and development but also play the important role in formation of plant immunity, in particular, take effect on the synthesis of ethylene, salicylic and jasmonic acids, etc. [5, 7].

As is shown below, the selected strains of streptomycetes *S. avermitilis* IMV Ac-5015, *S. netropsis* IMV Ac-5025 and *S. violaceus* IMV Ac-5027 are characterized by the complex of practically useful properties, i.e. by antagonism to phytopathogens, by stimulating and protective effect on plants. Biopreparations Avercom, Phytovit and Violar were elaborated on the basis of metabolites of the above streptomycetes.

The highest total content of sterols was revealed in Avercom (9.48 mg/l), that was 1.2- and 5.5-fold higher than in Phytovit and Violar, respectively.

Table 2. Content of sterols in biopreparations on the basis of metabolites of soil streptomycetes

Sterols	Content of sterols in biopreparations mg/l		
	Avercom	Phytovit	Violar
Squalene	nd	0.14 ± 0.05	0.03 ± 0.01
Cholesterol	1.69 ± 0.33	0.05 ± 0.01	0.02 ± 0.01
Ergosterol	2.13 ± 0.39	5.94 ± 0.71	1.29 ± 0.23
Sitosterol	nf	0.28 ± 0.09	0.25 ± 0.09
Stigmasterol	nf	0.06 ± 0.02	0.05 ± 0.02
24-epibrassinolide	5.66 ± 0.69	0.58 ± 0.15	0.07 ± 0.02
Total content	9.48 ± 1.03	7.85 ± 0.93	1.71 ± 0.44

Note: "nd" – not determined, "nf" – not found

In our opinion the use of biological preparations, containing sterols of microbial origin, can change their balance in a plant that, undoubtedly, is of great importance in interrelation of a host-plant and phytopathogen, and can affect the level of plant resistance to phytopathogens. In our previous researchers this admission was confirmed in field experiments with the wheat, tomato, Chinese cabbage, etc. The use of exogenic sterols in a complex with other biologically active components included in biopreparations, based on the studied streptomycetes, favored the increase of plants resistance to the broad spectrum of the agents of root rots, fusarioses, phytophthoroses, nematodoses that, as a result, led to the increase of harvest and improvement of quality of the obtained production.

Thus, we have first determined the ability of soil streptomycetes to synthesize in various independent ways the broad spectrum of sterols, squalene being their commom precursor. The use of metabolic biopreparations developed on the basis of the studied streptomycetes, containing sterols of microbial origin, is significant for regulation of the steroid pool in plants and increase of their resistance to phytopathogens and abiotic stresses, including the negative effect of pesticides.

БІОСИНТЕЗ СТЕРОЛІВ ҐРУНТОВИМИ СТРЕПТОМІЦЕТАМИ

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Метою роботи було дослідження біосинтезу стеролів ґрунтовими стрептоміцетами *Streptomyces avermitilis* IMB Ac-5015, *Streptomyces netropsis* IMB Ac-5025 і *Streptomyces violaceus* IMB Ac-5027 на різних середовищах за глибинного вирощування та визначення їх вмісту в біопрепаратах, розроблених на основі метаболітів цих продуцентів. Аналіз дериватів стеролів, екстрагованих із біомаси, супернатантів культуральних рідин та біопрепаратів, проводили методом GC/MS. У біомасі стрептоміцетів стероли було виявлено в значно більшій кількості, ніж у супернатантів культуральних рідин, а їхній спектр і співвідношення різнилися. Сквален (попередник стеролів) виявлено в біомасі продуцентів у кількості 16,2–43,3 мкг/г у разі вирощування їх на синтетичному і

8,2–212,1 мкг/г – на органічному середовищах. У біомасі *S. avermitilis* переважав 24-епібрасинолід, вміст якого був найвищим серед досліджуваних штамів і сягав 268,2 мкг/г на органічному і 345 мкг/г на синтетичному середовищах. *S. avermitilis* не синтезував ситостерол і стигмастерол, що є важливим з огляду на нематоцидні властивості штаму. У біомасі *S. netropsis* і *S. violaceus*, вирощених на органічному середовищі, вміст ситостеролу та стигмастеролу був вищим порівняно із синтетичним середовищем у 2,3–6,8 і у 1,5–3,0 рази відповідно. У складі стеролів *S. netropsis* і *S. violaceus* переважав ергостерол. У метаболічних біопрепаратах найбільший загальний вміст стеролів (9,5 мг/л) виявлено в Аверкомі (продуцентом є *S. avermitilis* IMB Ac-5015). Використання екзогенних стеролів мікробного походження важливе для регулювання їх співвідношення в рослинах та підвищення резистентності до фітопатогенів і фітонематод.

Ключові слова: ґрунтові стрептоміцети, стероли, індуктори стійкості рослин, фітопатогени, фітонематоди.

БИОСИНТЕЗ СТЕРОЛОВ ПОЧВЕННЫМИ СТРЕПТОМИЦЕТАМИ

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Целью работы было изучение биосинтеза стеролов почвенными стрептомицетами *Streptomyces avermitilis* ИМВ Ac-5015, *Streptomyces netropsis* ИМВ Ac-5025 и *Streptomyces violaceus* ИМВ Ac-5027 на различных средах при глубинном выращивании и определение их содержания в биопрепаратах, разработанных на основе метаболитов этих продуцентов. Анализ дериватов стеролов, экстрагированных из биомассы, супернатантов культуральной жидкости и биопрепаратов, проводили методом GC/MS. В биомассе стрептомицетов стеролы были обнаружены в значительно большем количестве по сравнению из супернатантами культуральных жидкостей, а их спектр и количественное соотношение отличались. Сквален (предшественник стеролов) был обнаружен в биомассе продуцентов в количестве 16,2–43,3 мкг/г при выращивании стрептомицетов на синтетической и 8,2–212,1 мкг/г на органической средах. В биомассе *S. avermitilis* ИМВ Ac-5015 преобладал 24-эпибрассинолид, содержание которого было самым высоким среди исследуемых штаммов и достигало 268,2 мкг/г на органической и 345 мкг/г синтетической средах. *S. avermitilis* не синтезировал ситостерол и стигмастерол, что является важным, учитывая нематоцидные свойства штамма. В биомассе *S. netropsis* и *S. violaceus*, выращенных на органической среде, содержание ситостерола и стигмастерола выше по сравнению с биомассой, выращенной на синтетической среде в 2,3–6,8 и в 1,5–3,0 раза соответственно. В составе стеролов *S. netropsis* и *S. violaceus* преобладал эргостерол. В метаболитных биопрепаратах наибольшее общее содержание стеролов (9,5 мг/л) обнаружено в Аверкоме (продуцент *S. avermitilis* IMB Ac-5015). Использование экзогенных стеролов микробного происхождения важно для регулирования их соотношения в растениях и повышения резистентности к фитопатогенам и фитонематодам.

Ключевые слова: почвенные стрептомицеты, стеролы, индукторы устойчивости растений, фитопатогены, фитонематоды.

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